# D4.2

# Methodological manual

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# Executive Summary

This Methodological Manual describes in detail the methodological approaches that will be used in Work Package 4 (“Climate mitigation services and C and GHG processes in wetlands”) of the project RESTORE4Cs. More specifically, this manual presents the methodology i) to conduct the literature review and context setting on carbon (C) storage capacity and greenhouse gas (GHG) fluxes in coastal wetlands, ii) to choose the subsites within each pilot case where fieldwork will be conducted, iii) to define in detail the sampling protocol and subsequent laboratory analyses that will  provide key datasets to characterize the C storage capacity, GHG fluxes, and the ecological status of each subsite, iv) to describe how the LUPLES method (Land Uses for estimating Pressure LEvelS) will be refined.

# List of abbreviations

| | |
|---|---|
| C | Carbon |
| GHG | Greenhouse gas |
| GC | Gas chromatography |
| LUPLES | **L**and **U**se- - **P**ressure **L**evel – **E**cological **S**tatus |

# 1. Introduction

RESTORE4Cs seeks to evaluate how restoration efforts impact the ability of wetlands to mitigate climate change and provide various ecosystem services by adopting a comprehensive socio-ecological systems approach. Focusing on European coastal wetlands, RESTORE4Cs will establish uniform methods and strategies for prioritizing restoration that enhances carbon (C) storage, reduces greenhouse gas (GHG) emissions and radiative forcing, and enhances ecological health, as well as offering additional benefits like flood control and coastal erosion protection.

Within this framework, Work Package 4 (WP4) covers the "Climate mitigation services and C and GHG processes in wetlands". WP4 aims at overcoming knowledge and data gaps on C-storage capacity and GHG emissions mitigation from well-preserved, altered and restored coastal wetlands. First, a literature review and context overview are provided (task 4.1, deliverable D4.1), presenting the current state of the art on the effect of wetlands restoration to enhance climate mitigation services. This first review serves as a basis to develop an extensive meta-analysis (WP5). Then, six sites for each of the six pilot sites selected for RESTORE4Cs are chosen (task 4.2, D4.1), leading to 36 sampling sites where *in-situ* data will be collected. This fieldwork effort will provide crucial data on key wetland ecological indicators (task 4.3) and C storage, GHG fluxes (task 4.4) to feed modelling and upscaling approaches (WP3, WP7).

The present manual describes in detail the methodological approaches chosen for the preliminary literature review and context setting (section 2.1) and the meta-analysis (section 2.2). Then, section 3 provides the rationale for the choice of the subsites. Section 4 contains an overview of the sampling approach, including the list of variables, including GHG fluxes, ecological indicators, and ancillary data to be measured *in-situ*). The aim of this manual is also to serve as a guide for the fieldwork team, solving methodological and logistical issues. Further, the manual can be exchanged with other sister projects that are measuring similar ecological variables and processes, in such a way that it will facilitate interoperability among the results obtained in the different sister projects. Therefore, all the sampling protocol for the fieldwork and all subsequent laboratory analyses are given in extensive details in section 5. Finally, section 6 describes how the LUPLES method (Land Uses for estimating Pressure LEvelS) will be refined within the frame of the project.

# 2. Methodology for literature review and meta-analysis

## *2.1. Approach for literature review and preliminary meta-analysis for context overview*

A first review of the literature for a general context overview (deliverable 4.1) aimed to first identify main countries and developments over time related to the research on coastal wetland restoration and carbon (C) stocks and fluxes, and then to compare C stocks and greenhouse gases (GHG) fluxes at altered versus restored sites in coastal wetlands (preliminary meta-analysis). To extract the literature on coastal wetlands, their management, and the C cycle within them, we relied on the literature repository Web of Science (Clarivate, *www.webofscience.com*). All used search queries are summarized in Table 1, including the date the search was run and for which analysis they were utilized. The code listed in Table 1 is used in the following text to refer to the specific search queries.

*Table 1. Search queries in Clarivate Web of Science and their parameters used for the literature review (deliverable D4.1).*

| Code | Search query | Date of search | Aim |
|---|---|---|---|
| (1) | *coastal* AND *wetland* (Topic) | 21.03.2023 | Identification of global and European research hotspots |
| (2) | *coastal* AND *wetland* AND *restoration* (Topic) | 21.03.2023 | Identification of global and European research hotspots |
| (3) | *coastal* AND *wetland* AND *restoration* AND (ecosystem service* OR *biodiversity* OR *carbon* OR *flood* OR *nutrient*) (Topic) | 21.03.2023 | Assessing development of coastal wetland research over time |
| (4) | *coastal* AND *wetland* AND (ecosystem service* OR *biodiversity* OR *carbon* OR *flood* OR *nutrient*) (Topic) | 21.03.2023 | Assessing development of coastal wetland restoration research over time |
| (5) | *coastal* AND *wetland* AND *restoration* AND *carbon* (Topic) | 21.03.2023 | Assessing importance of carbon research among coastal wetland restoration research |

| (6) | *coastal* AND *wetland* AND *europ* AND *carbon* (Topic) | 09.03.2023 | Data provision for meta-analysis |
|---|---|---|---|
| (7) | *coastal* AND *wetland* AND *europ* AND (GHG OR CO2 OR CH4 OR N2O) (Topic) | 09.03.2023 | Data provision for meta-analysis |
| (8) | *coastal* AND *wetland* AND (GHG OR CO2 OR CH4 OR N2O) AND *restoration* (Topic) | 09.03.2023 | Data provision for meta-analysis |

To identify global and European research hotspots, the country where the research was published was exported for search queries (1) and (2). To assess the development of wetland restoration research over time, the number of publications per year was exported for search queries (3), (4), and (5). To keep the data comparable over the years, only full years were used, and 2023 was excluded. For the preliminary meta-analysis, we used search queries (6), (7), and (8). As the total number of publications about the C cycle and GHGs in global wetlands was too high for a preliminary analysis, these search queries ((6) & (7)) were focused on European studies. Considering the framework of RESTORE4Cs we started with a search focused on Europe only ((6) & (7)). Due to a lack of studies covering restoration effect on GHG in Europe we conducted an additional search query for GHG not only limited to Europe (8).

The search queries (6), (7), and (8) combined resulted in 110 publications (Figure 1). After a screening of the retrieved publications to remove for our purpose irrelevant research and to separate them into review articles and publications reporting original data, data from 51 publications were extracted. The resulting data table will be the foundation for the planned meta-analysis in Task 4.5. For this meta-analysis, we selected only studies comparing altered with restored systems, reporting mean values, SD or SE, and the number of replicates taken, which resulted in 13 publications. These studies contain data about C stocks, $CO_2$ fluxes, $CH_4$ fluxes, and $N_2O$ fluxes. No study was found reporting carbon sequestration rates in the necessary format described above. The meta-analysis was run separately for the four studied parameters. Calculations for the meta-analysis were done with R (R Core Team, 2022) and the "metafor" package (Viechtbauer, 2010), using standardized mean difference as method (Hedges, 1981).

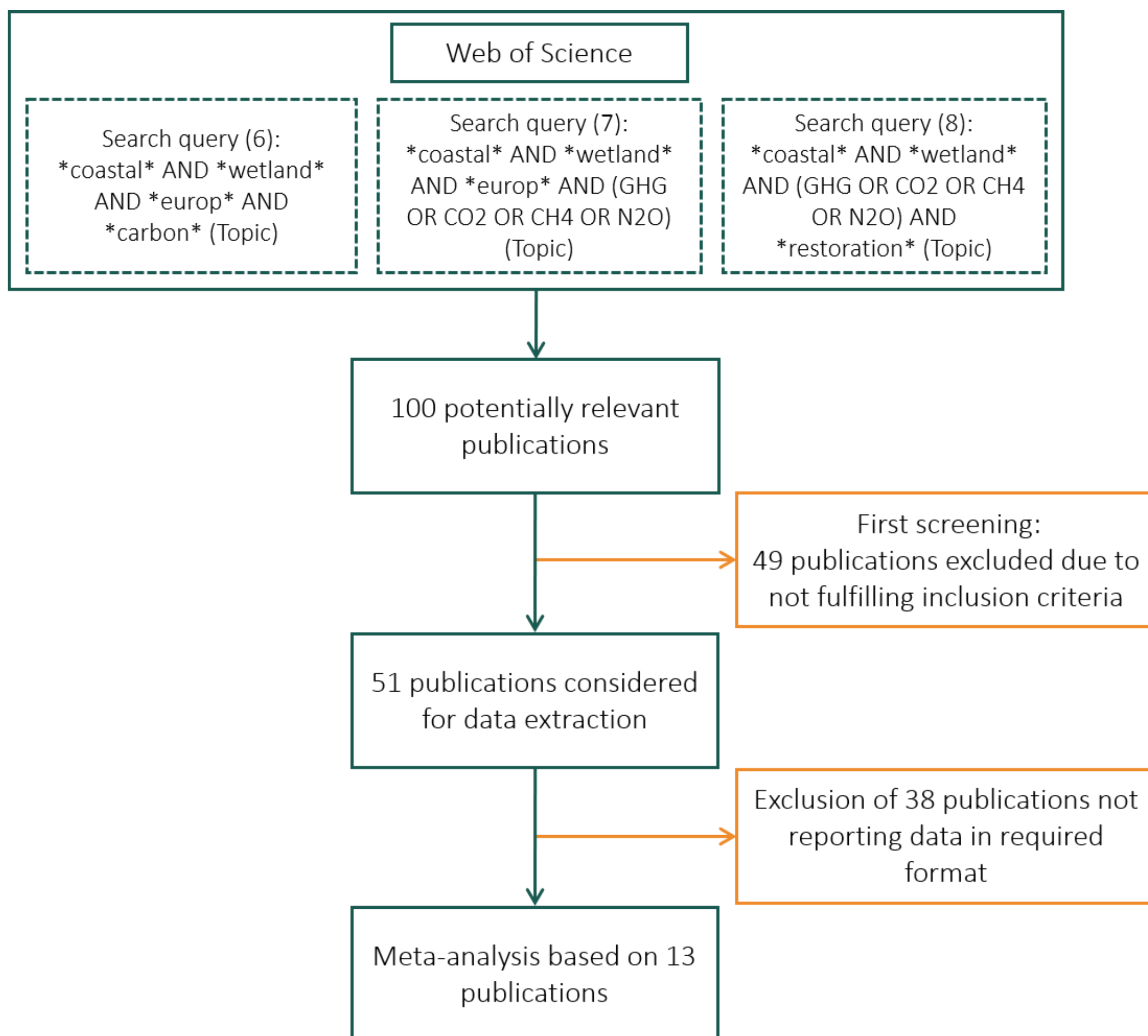


*Figure 1. Schematic overview of the workflow of literature selection for the preliminary literature-review meta-analysis (Task 4.1).*

## 2.2. Approach for the in-depth meta-analysis for task 4.5

Since, an in-depth meta-analysis is yet to be done, contrastingly with the literature review previously reported, the procedures described here are presented in future tense. After a bibliographic search targeting defined terms, including specifically restoration and rehabilitation on coastal wetlands and C and GHG exchanges, a selection of the studies and the sites with enough information to carry out the analysis will be made. For that, a definition of the criteria to select or discard studies in function of their characteristics and approaches will be performed. Data will be extracted from the main text or tables and figures, also from supplementary material when possible.

Data will be ordered to classify the included ecosystems in different categories and to proceed with their statistical analyses. For that, a definition of the conservation status, according to

some criteria will be previously defined. As studies will have different information, procedures to homogenise the status will be established, to include all the sites in different categories or ranges for the statistics.

A compilation of different parameters related to the carbon cycle (e.g., metabolic rates, C and GHG fluxes, C stocks) will be carried out, and normalized, grouped by wetlands habitats/ecosystem types, statuses, and types of alterations and restorations. Depending on the information and the way it will be compiled, the best statistic tests to perform the meta-analysis and explore the hypothesis raised will be defined. Response ratio calculations will be performed, differentiating between landmarks (reference sites) and restored or altered places, to assess, together with the data compiled in the case pilot studies, the effectiveness of the restoration actions recovering the ecosystem health.

## References

Hedges, L. V. (1981). Distribution Theory for Glass's Estimator of Effect Size and Related Estimators. *Journal of Educational Statistics*, *6*(2), 107–128. https://doi.org/10.2307/1164588

R Core Team. (2020). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Viechtbauer, W. (2010). Conducting meta-analyses in R with the metafor package. *Journal of Statistical Software*, *36*(3), 1–48.

# 3. Subsite selection

For the RESTORE4Cs project, coastal wetlands at six Case Pilots study sites have been selected: Ebro Delta/Valencian wetlands (Mediterranean), Camargue (Mediterranean), Ria de Aveiro (Atlantic), Oosterschelde/Grevelingen Delta (Atlantic), Curonian Lagoon (Baltic) and Danube Delta (Black Sea). We selected six subsites within each Case Pilot site where the field sampling will be conducted. The process of the subsite selection is described in the following paragraphs.

The selection of subsites consists of the following steps:

1) Identification of relevant criteria for subsite selection
2) Revision of selection criteria
3) Proposal of potential subsites by partners
4) Final discussion and subsite selection

The first version of the subsite selection criteria was shared with the project partners. After all the partners revised the subsite selection criteria, the following criteria were defined:

- Only habitat/ecosystem types should be proposed, which allow the sampling of **three types of subsites**, and at least two subsites per type (6 sampling subsites in the habitat/ecosystem type in total), as follows:
    - **2 Well-preserved sites:**
      Should represent the subsites of the wetland habitat/ecosystem types where no significant anthropogenic modifications occurred. Only natural processes are observed, with no significant alterations. For RESTORE4Cs, the concept of well-preserved is similar to that of the good conservation status for the structure & function and future prospects at the subsite level (Habitats Directive) and to the good ecological status at the subsite level (WFD);
    - **2 Altered sites:**
      The ecosystem has lost its main structural and functional ecological attributes (e.g., seagrass beds losing seagrasses);
    - **2 Restored sites:**
      Sites returned to reference (similar to those of the well-preserved sites) conditions (e.g., seagrasses restored).

- **Alterations** should be of one of the following types
    - Hydrological (reductions in the normal volume, over-damming by a heightening of the waterbody basin)

    - Morphological (altered by modifications or constructions)
    - Trophic (mainly due to land use effect) – eutrophication & organic enrichment
    - Other pollution types (e.g., acidification, xenobiotics, heavy metals; specified by partners)
    - Mixed (specified by partners)
    - Any other (specified by partners)

- The **wetland restoration practice** might be:
    - **Active restoration** that eliminates the source of degradation and disturbance of an ecosystem and implements measures to accelerate its recovery and overcome obstacles to that recovery
    - **Passive restoration** - eliminates the factors of degradation and disturbance and permits the natural regeneration of the ecosystem
- The **wetland restoration actions** adopted might be:
    - related to soils
    - related to hydrology
    - related to water quality
    - related to vegetation
    - related to morphological reconstruction
    - any other (specified by partners)
- The proposed subsites should be **accessible for sampling**, including required permissions.

- **Basic ecological data** on the ecology of the subsites should be available:
  - C and GHG
  - Biological
  - Abiotic
  - Social
  - Economic
  - Other (specified by partner)
- **More detailed information**, both ecological (WP4) and socio-economic (WP2, WP5), is needed on the Case Pilot site as a whole.

After subsite selection criteria were defined, each Case Pilot partner was asked to fill in the file template (Figure 2) to indicate all habitat types (see Table 1 in Misteli et al., 2023) fulfilling all selection criteria. They were asked to list as many habitat types as possible in order of distribution (% of the total wetland area).

## *References*

Misteli, B., Attermeyer, K., Minaudo, C., von Schiller, D, Obrador, B., Abdul Malak, D., Sánchez, A., Coelho, J.P., Sousa, A., Morant, D., Rochera, C., Vaičiūtė, D., Kataržytė, M., Bučasm, M., Petkuvienė, J., Čerkasova, N., Guelmami, A., Anglada, C., Lago, M., Walles, B., Cazacu, C., Camacho, A. (2023). Case Pilots overview and context setting. Deliverable D4.1 Horizon RESTORE4Cs Project GA ID: 101056782

**CASE PILOT:** insert here the name of your case pilot

**PARTNER IN CHARGE:** insert here the name of your case pilot

**PERSON IN CHARGE AS THE LOCAL PARTNER:** insert here the name of your case pilot

| HABITAT TYPE & REPRESENTATIVENESS (% of the local wetland area at case pilot level) | Status | Geographic coordinates N | Geographic coordinates E-W | The area size, sq. m. | Type of alteration, level (weak, intermediate, high, destroying the habitat) and time since alterations ceased | Type of restoration and time from the end of the restoration actions | Type of restoration measures | Distance from the lab where samples can be processed | Type and amount of information available on the subsites | Additional subsites for eventual replacement | Facilities (Lab available in the vicinity, accomodation, etc.) | Any other information |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. - Freshwater marshes and ponds<br>2. Brackish marshes<br>3.- Intertidal salt marshes<br>4.- Microtidal salt marshes<br>5. - Freshwater ponds<br>6.- Brackish ponds<br>7.- Saline ponds<br>8.- Humid dune slacks<br>9.- Submerged plant beds<br>10.- Reed beds.<br>11.- Intertidal seagrass beds<br>12.- Karstic springs<br>13. Any other (specify) | 1 .- Well preserved<br>2.- Altered<br>3.- Restored | | | | 1 - Hydrological 2- Morphological 3 - Trophic 4. Other pollution: acidification, xenobiotics, heavy metals, etc. (specify) 5 - Mixed (specify) 6 - Other (specify) | 1. Active restoration that eliminates the source of degradation and disturbance of an ecosystem and implements measures to accelerate its recovery and overcome obstacles to that recovery.<br>2. Passive restoration - eliminates the factors of degradation and disturbance and permits the natural regeneration of the ecosystem. | 1. related to soils<br>2. related to hydrology<br>3. related to water quality<br>4. related to vegetation<br>5. related to morphological reconstruction<br>6. Any other (specify) | Km and time by car in hours | 1. C and GHG<br>2. Biological<br>3. Abiotic<br>4. Social<br>5. Economic<br>6. Other (specify) | Number per status,and coordinates | | |
| Insert here the name of your habitat type -1 | 1 .- Well preserved | | | | | | | | | | | |
| Insert here the name of your habitat type -1 | 1 .- Well preserved | | | | | | | | | | | |
| Insert here the name of your habitat type -1 | 2.- Altered | | | | | | | | | | | |
| Insert here the name of your habitat type -1 | 2.- Altered | | | | | | | | | | | |
| Insert here the name of your habitat type -1 | 3.- Restored | | | | | | | | | | | |
| Insert here the name of your habitat type -1 | 3.- Restored | | | | | | | | | | | |

*Figure 2. Excel sheet example used for subsites selection that each Pilot Site leaders had to fill in.*

# 4. Fieldwork – general overview

This Section describes the fieldwork approach, providing an overview to the timing of the sampling campaigns, and listing the different compartments and variables that will be sampled at each subsite. For the detailed sampling and analytical protocols, please refer to Section 5.

## 4.1. *Timing, samples and measurements, and overall sampling approach*

Project RESTORE4Cs relies on the *in-situ* characterization of key physical, biogeochemical and biological variables. Sampling campaigns will be conducted at each subsite every three months, for an entire year (starting in October 2023, January 2024, April 2024, and July 2024).

To reach the project objectives, at each subsite, samples and measurements will be taken from the atmospheric, aquatic, benthic, vegetated, bare soil or sediment compartments. Figure 3 provides an overview of all the variables that will be measured in each of these compartments and at the interfaces between air-water and air-sediment/soil.

Some of these variables are key to the project and directly related to GHG fluxes, either measured in-situ or through incubation experiments, as explained in section 4.2.

Additionally, a selection of ecological variables was generated to feature and assess the ecological and conservation status of the pilot sites, as described in section 4.3. These variables also characterise the types of alterations and the restoration actions implemented at each site. They correspond to classic physical and chemical features of both waters and sediments, as well as atmospheric conditions and indicators for different biological communities, including phytoplankton, benthic invertebrates, zooplankton, bacteria, and archaea.

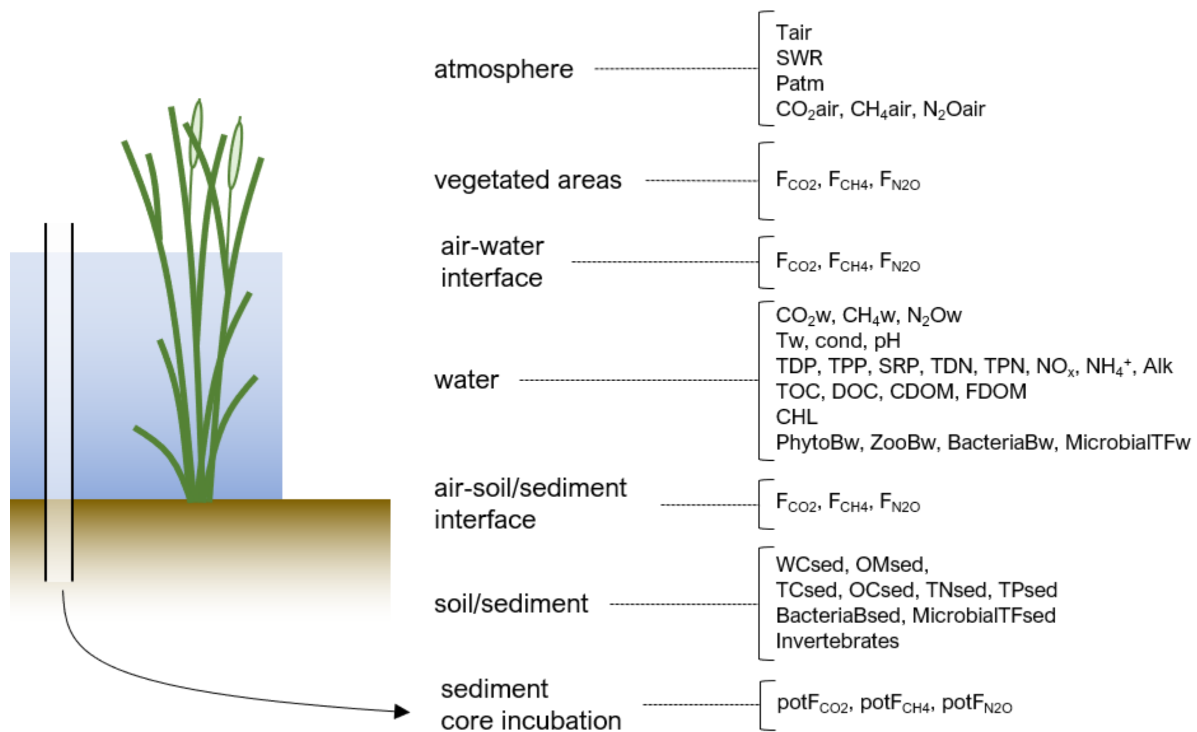


*Figure 3. Schematic overview of the different compartments and variables that will be measured at each subsite. For a description of each variable, refer to Tables 3 and 4.*

Our sampling approach involves direct *in-situ* measurements, sediment core incubations, and taking samples in the field for subsequent analyses in the laboratories of RESTORE4C's project partners. Most samples need to be processed or prepared before being sent for analysis to the different laboratories. These steps are detailed in the protocols in Section 5. All samples requiring further processing for analyses will be distributed to the different project partners, according to their expertise and analytical facilities. The following table (Table 2) lists all the variables where samples will be transferred to after each sampling campaign.

*Table 2 Repartition of the different lab analyses among project partners.*

| Variables (method) | Institution and contact person |
| --- | --- |
| CO2w, CH4w, N2Ow<br>CO2air, CH4air, N2Oair<br>(gas chromatography) | University of Barcelona (UB, Spain)<br>Camille Minaudo (camille.minaudo@ub.edu)<br>Daniel von Schiller (d.vonschiller@ub.edu)<br>Biel Obrador (obrador@ub.edu) |
| Sediment analyses: WC, OMsed, TCsed, OCsed, TNsed, C+N stable isotopes<br>(ignition, elemental analysis) | WasserCluster Lunz (WCL, Austria)<br>Katrin Attermeyer (katrin.attermeyer@wcl.ac.at)<br>Benjamin Misteli (benjamin.misteli@wcl.ac.at) |
| Sediment analyses: TPsed<br>(ignition and acid effect + spectroscopy) | University of Bucharest (UNIBUC, Romania)<br>Constantin Cazacu (constantin.cazacu@g.unibuc.ro)<br>Mihai Adamescu (mihaicristian.adamescu@g.unibuc.ro) |

| | |
|---|---|
| Nutrients concentrations: SRP, TPP, NH4, NO3+NO2, TPN (APHA)<br>Benthic invertebrates (marine) biomass and composition<br>(microscopy) | University of Aveiro (UAveiro, Portugal)<br>Joao Pedro Coelho (jpcoelho@ua.pt)<br>Heliana Teixeira (heliana.teixeira@ua.pt) |
| Zooplankton biomass and composition<br>Benthic invertebrates (freshwater) biomass and composition<br>(microscopy) | Tour du Valat (TdV, France)<br>Anis Guelmami (guelmami@tourduvalat.org)<br>Samuel Hilaire (hilaire@tourduvalat.org) |
| Alkalinity (titration)<br>Bacterial abundance in the water and the sediment (flow cytometry)<br>Taxonomic and functional microbial diversity in the water and the sediment (DNA sequencing and functional annotation) | University of Valencia (UVEG, Spain)<br>Carlos Rochera (carlos.rochera@uv.es)<br>Antonio Camacho (antonio.camacho@uv.es)<br>Antonio Picazo (antonio.picazo-mozo@uv.es)<br>Javier Miralles (javier.miralles-lorenzo@uv.es)<br>Daniel Morant (daniel.morant@uv.es)<br>Rafael Carballeira (rafael.carballeira@uv.es) |
| TOC, DOC (HTCO)<br>CDOM, FDOM (Fluorescence and absorption spectrometry) | Consiglio Nazionale della Ricerche (CNR, Italy)<br>Chiara Santinelli (chiara.santinelli@ibf.cnr.it) |
| Photosynthetic pigments: chlorophylls and taxa specific carotenoids (HPLC) | Klaipeda University (KU, Lithuania)<br>Jolita Petkuvienė (jolita.petkuviene@ku.lt)<br>Diana Vaičiūtė (diana.vaiciute@jmtc.ku.lt) |
| Phytoplankton biomass and composition (microscopy) | University of Salento, LifeWatchERIC (UNILE, Italy)<br>Jessica Titocci (jessica.titocci@iret.cnr.it)<br>Alberto Basset (alberto.basset@unisalento.it) |

## 4.2. In-situ and sediment core incubations for GHG fluxes assessment

GHG fluxes to the atmosphere will be quantified at the subsite level with two different and complementary approaches:

Direct *in-situ* measurements will be carried out using discrete air and water samples, and chambers connected to portable gas analysers, allowing to estimate fluxes for inundated, vegetated and bare soil or sediment areas ($F_{CO2}$, $F_{CH4}$, $F_{N2O}$).

Sediment cores will be taken in inundated areas, including water layer and air headspace, and transported to the laboratory for standardized incubation and assessment of potential fluxes (pot$F_{CO2}$, pot$F_{CH4}$, pot$F_{N2O}$).

Chamber measurements allow for the quantification of real *in-situ* fluxes and enable to assess spatial heterogeneities. These measurements will be key to upscaling GHG fluxes estimation at larger scales, from the subsite to entire pilot site scale, and to extrapolating to pan-European wetlands. Yet, chamber measurements are sensitive to the local meteorological conditions, which could impede a robust comparison of GHG fluxes across different subsites. To ensure comparable fluxes estimates across different subsites with different degrees of ecological

alteration or restoration, incubation of sediment cores from different subsites placed under standardized conditions will provide crucial estimates of potential GHG fluxes.

Table 3 presents the exhaustive list of variables to be measured *in-situ* or in the laboratory for GHG fluxes assessment.

*Table 3. Variables to be measured in-situ or in the laboratory for GHG fluxes assessment This table also indicates for each variable the corresponding Table index and pages in the Manual where to find the description of the sampling and analytical procedures.*

| Variable ID | Description | Table index and corresponding pages |
| --- | --- | --- |
| CO2air, CH4air, N2Oair | CO2, CH4 and N2O air concentrations | Table GHG.1 - pages 26-28 |
| CO2w, CH4w, N2Ow | CO2, CH4 and N2O water concentrations (headspace method) | Table GHG.1 - pages 26-28 |
| FCO2, FCO4, FN2O | *In-situ* CO2, CH4 and N2O fluxes | Table GHG.1 - pages 26-28 |
| potFCO2, potFCH4, potFN2O | Potential CO2, CH4 and N2O fluxes measured by sediment cores incubations | Table GHG.2 - pages 29-30 |

## *4.3. Ecological indicators and ancillary data*

Key ecological indicators were selected to feature the subsites and, mainly, to assess the ecological and conservation status of the pilot sites. These indicators also characterize the types of alterations, and the restoration actions implemented at each site. They correspond to physical and chemical features of both waters and sediments, as well as vegetation cover, atmospheric conditions, and indicators for different biological communities, including phytoplankton, benthic invertebrates, zooplankton, bacteria, and archaea.

For each pilot site and their respective subsites, the selected indicators aid in characterizing the C stock and storage capacity of the different habitats, as well as the trophic status (nutrient levels), metabolic rates (production/respiration), and the abundance and biodiversity of aquatic taxa that are key for the C cycle in wetland ecosystems.

Table 4 presents the exhaustive list of indicators chosen and organized by type (biological/chemical/physical) and ecosystem compartment: air (white) / water (blue) / sediment (light yellow). This table also indicates for each indicator the corresponding Table index and pages in the Manual where to find the description of the sampling and analytical procedures.

*Table 4. Ecological indicators and ancillary data chosen to characterize the ecological status at each site and subsite in the water (blue shades), sediment (light yellow), and air (white) compartments. The last column indicates the corresponding Table number and pages in the Manual where to find the description of sampling and analytical procedure.*

| Indicator ID | Description | Compartment | Type | Objective: characterize.... | Table index and corresponding pages |
|---|---|---|---|---|---|
| TDP | Total dissolved phosphorus concentration | water | Chemical | trophic state | Table W.1<br>p 32-33 |
| SRP | Soluble reactive phosphorus concentration | water | Chemical | trophic state | |
| TPP | Total particulate phosphorus concentration | water | Chemical | trophic state | |
| TDN | Total dissolved nitrogen concentration | water | Chemical | trophic state | |
| TPN | Total particulate nitrogen concentration | water | Chemical | trophic state | |
| NH4 | Ammonium concentration | water | Chemical | trophic state | |
| NOx | Nitrate + nitrite concentration | water | Chemical | trophic state | |
| CHLA | Photosynthetic pigments (Chlorophylls and taxa specific carotenoids) | water | Biological | primary productivity and trophic state | Table W.2<br>p 33-34 |
| Alk | Alkalinity | water | Chemical | Amount of bicarbonates and carbonates | Table W.3<br>p 34-35 |
| TOC | Total organic carbon | water | Chemical | quantity of organic matter pool | Table W.4<br>p 35 |
| DOC | Dissolved Organic Carbon concentration | water | Chemical | quantity of dissolved organic matter pool | Table W.5<br>p 36 |

| | | | | | |
|---|---|---|---|---|---|
| CDOM | Chromophoric Dissolved Organic Matter | water | Chemical | quality of dissolved organic matter | Table W.6 p 36-38 |
| FDOM | Fluorescent Dissolved Organic Matter | water | Chemical | quality of dissolved organic matter | Table W.6 p 36-38 |
| BacteriaBw | Bacterial abundance | water | Biological | biomass of bacteria | Table W.7 p 38-39 |
| MicrobialTFw | Taxonomic and functional microbial diversity | water | Biological | composition and functional annotation of the prokaryotic community | Table W.8 p 39-40 |
| PhytoBw | Phytoplankton biomass, counts and indices | water | Biological | Phytoplankton abundance and diversity | Table W.9 p 40-41 |
| ZooBw | Zooplankton counts and indices | water | Biological | biomass and composition of zooplankton community | Table W.10 p 41-42 |
| Tw | Water temperature | water | Physical | thermal regime and normalize other variables | Section 5.1.a p 22 |
| Cond | Electrical conductivity | water | Physical | overall ionic balance | Section 5.1.a p 22 |
| z | Water depth a time of sampling | water | Physical | hydromorphology | Section 5.1.a p 22 |
| pH | pH | water | Chemical | water chemical properties | Section 5.1.a p 22 |
| Salinity | Salinity | water | Physical | water chemical properties | Section 5.1.a p 22 |
| DO | Dissolved Oxygen concentration | water | Chemical | Physiochemical properties | Section 5.1.a p 22 |
| WCsed | Water content | sediment | Physical | Humidity in the sediment | Table Sed.1 |

| | | | | | |
|---|---|---|---|---|---|
| OMsed | Organic matter content in the sediment | sediment | Biological | organic matter stock in the sediment | p 44 |
| TCsed | Total Carbon content in the sediment | sediment | Chemical | C stock in the sediment | Table Sed.2 p 44-45 |
| TNsed | Total nitrogen content in the sediment | sediment | Chemical | nitrogen stock in the sediment | |
| TPsed | Total phosphorus content in the sediment | sediment | Chemical | phosphorus stock in the sediment | Table Sed.3 p 45 |
| BacteriaBsed | Bacterial abundance | sediment | Biological | bacterial abundance in the sediments | Table Sed.4 p 46 |
| MicrobialTFsed | Taxonomic and functional microbial diversity | sediment | Biological | composition and functional annotation of the prokaryotic community in the sediments | Table Sed.5 p 47 |
| Benthic invertebrates | Benthic invertebrate biomass abundance and composition | sediment | Biological | benthic invertebrate community | Table Sed.6 p 48 |
| Tair | Air temperature | air | Physical | atmospheric conditions | Section 5.4.c p 49 |
| SWR | Short wave solar radiations | air | Physical | meteorological conditions | |
| Patm | Atmospheric pressure | air | Physical | meteorological conditions | |

# 5. Sampling protocol and subsequent analyses

This section provides detailed information on how the sampling will be performed. After some general considerations and recommendations in section 5.1, section 5.2 describes the protocol that will be followed for GHG fluxes quantification, and section 5.3 details the sampling and subsequent analytical methodology that will be employed for all the different ecological indicators and other ancillary variables.

## *5.1. General considerations*

### *a) Instruments*

- Testing, calibrating with standards and inter-comparison of instruments are basic steps that must be included.
- The operators of the instrument must be trained and have the required competence in making the analysis.
- Each instrument's maintenance specifications need to be followed. Most instruments need to be zeroed, calibrated and warmed up before use. Zeroing and calibration can be performed by the user or by the manufacturer, depending on the instrument. The warm-up period can vary greatly between instruments and should be respected.

### *b) Labelling samples*

All samples must be coded and labelled following the same nomenclature, as follows:

[site and subsite ID] – [sampling campaign] – [date] – [replicate ID] – [variable]

- subsite ID: pre-determined unique subsite identifier, which also indicates the pilot site.
- sampling campaign: number, 1 to 4 campaigns, with "S" as a prefix.
- date: date of sampling in the YYMMDD format. Time of sampling using UTC time as a reference will be consigned in pre-prepared forms and field notebooks.
- replicate ID: identifier for replicate within a subsite, with "r" as a prefix. Exact geographic coordinates of the sampling point for each replicate must be consigned in the field notebook or pre-prepared form (see section 5.1.c).
- variable: variable name, in short

For instance, a correct identifier for a vial corresponding to SRP analysis, replicate r2, taken on 25 October 2023 at 13:35 at subsite CUR3 in the Curonian Lagoon pilot site is:

CUR3-S1-20231025-r2-SRP

### c) Reporting geographical coordinates

Geographical coordinates of all sampling points will be retrieved using the Google Maps application on mobile phones.

- Before sampling and if not yet done: download Google Maps app from app store.

On Android:

- tap and hold a location on the map to show latitude and longitude in the Search bar.
- tap the coordinates in the search bar at the top of the screen and select Copy. To paste, tap and hold the desired location and select Paste.

On iPhone:

- tap and hold a map location, then drag the information panel up to view latitude and longitude.
- tap the coordinates to instantly copy them to the clipboard. Then, tap and hold any typing area and select Paste.

The first coordinate is latitude and the second coordinate is longitude. Latitude/Longitude coordinates should be reported in decimal degrees.

### d) Order of sampling during fieldwork

The most sensitive measurements and samples must be taken first, leaving the most invasive techniques to the end. When sampling in open water areas, samples and measurements should be taken from a boat. The following order should be respected:

i) deployment of sensors (multiparametric probe);

ii) water sampling for all the ecological indicators and ancillary data derived from the water compartment;

iii) GHG chamber measurements;

iv) sediment cores, and additional sediment samples.

## 5.2. In-situ GHG fluxes

Each subsite will be stratified into three simple land cover strata based on areal contributions from (i) open water areas (more than 10 cm water depth, no emergent vegetation, regardless of submerged vegetation), (ii) vegetated areas (aquatic emergent, submerged with less than 10 cm water depth, or terrestrial, regardless of functional traits or biodiversity), and (iii) bare areas (soil or sediment exposed to the atmosphere, no apparent vegetation) exposed to the atmosphere. To ensure spatial representativeness, the total number of chamber measurements will be proportionately distributed among the three different strata (see a schematic example displayed in Figure 4), with a strict minimum of 3 chamber measurements per stratum, unless it represents less than 10% of the total area of the subsite. In the latter

case, a larger and more representative number of observations will be distributed to the two other strata. Then, sampling location will be randomized spatially for each stratum, allowing for a fair and objective distribution of the sampling plots among the subsites. The **stratified weighted randomized sampling** will be prepared in advance prior to sampling day and reiterated for each sampling campaign.

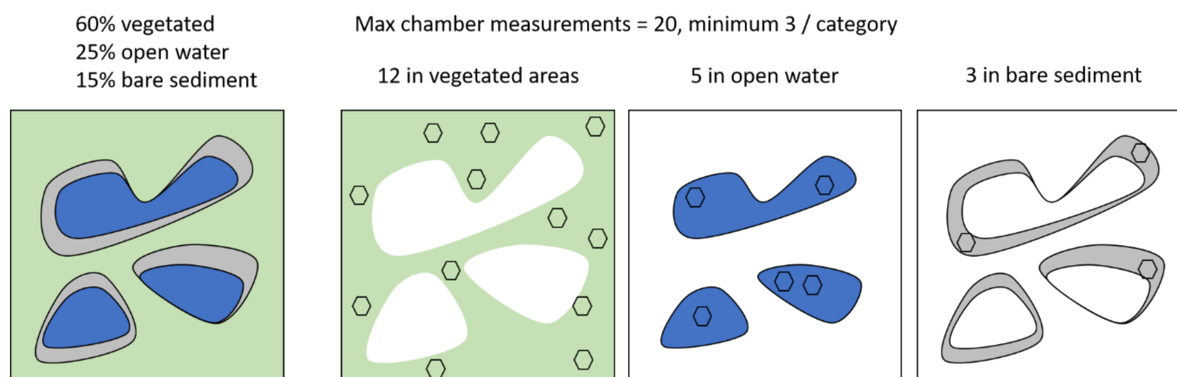


*Figure 4. Schematic example of the stratified sampling approach that will be implemented for GHG measurements. In this example, the virtual site is 60% vegetated, 25% covered by open water, and 15% bare sediments exposed to the atmosphere. Sampling plots, where chamber measurements are targeted, are distributed proportionally to each stratum.*

Discrete plots at each site will be pre-defined numerically based on the local cartography (e.g., shapefile) provided by pilot site partners, and using for instance the tiler R package (*https://cran.r-project.org/web/packages/tiler/vignettes/tiler-intro.html*) to spatialize at each subsite plots of equi-area. Spatial randomization will be ensured with the use of true randomizers, such as *https://www.random.org/integer-sets/*.

At each plot, *in-situ* GHG fluxes will be measured using two methods:

- The direct method: following gas concentration within floating or static chambers over time. For $CO_2$ and $CH_4$, this will be done with chamber measurements connected to portable infrared gas analysers (LICOR-7810 and a Picarro GasScouter G4301). For $N_2O$, this will be done by discrete gas sample of the air trapped in the chambers and subsequent gas chromatography (GC) analyses in the laboratory.
- The indirect method: measuring gas concentration in the air and the water (headspace + GC) for all three gases. Then, fluxes can be estimated using Fick's law of gas diffusion.

One advantage of the direct method is that it also may capture ebullition (or bubbling) of $CH_4$.

Air samples will be taken with triplicates at each subsite.

Water headspace samples will be taken without any replicate before each open water chamber measurement.

The measurement of GHG fluxes with gas chambers relies on the high-frequency monitoring of gas partial pressure in an enclosed chamber. For each chamber measurement, the gas flux is calculated as follows:

$$F_{gas} = \frac{dp_{gas}}{dt}\frac{V}{RST}$$

where $F_{gas}$ is expressed in [mmol m$^{-2}$ day$^{-1}$], $\frac{dp_{gas}}{dt}$ is the rate of change of the gas partial pressure within the chamber (positive when emission, negative when uptake) [µatm s$^{-1}$], $V$ is the volume of the chamber [m$^3$], $R$ is the ideal gas constant in [L atm K$^{-1}$ mol$^{-1}$], $T$ is the air temperature [K], $S$ is the area of the chamber [m$^2$].

A maximum of 15-20 gas chamber measurements is realistic at each subsite, given that each chamber measurement takes about 5 minutes for vegetated or bare sediment areas, and 10-15 minutes in open water areas (more than 10 cm depth) to capture both diffusive and ebullitive fluxes. Dark chambers will be used to avoid excessive heat accumulation during the measurements which are possibly impacting the results (Bastviken et al., 2015), except for vegetated areas where chamber measurements will be performed with both dark and transparent chambers (hence, 2 times 5 minutes measurements) to assess the effect of photosynthesis on the overall GHG fluxes.

For each chamber measurement, it is needed to record air temperature and incoming solar radiation inside the chamber, atmospheric pressure, exact start time and end time of measurement (using the time zone that the GHG analyser uses and note the time zone).

Additionally, local wind speed (U10) can be useful for upscaling GHG fluxes over time and record local meteorological conditions during the sampling. This ancillary data might rely on nearby meteorological stations.

Table GHG.1 below presents in detail the sampling and experiment procedure for *in-situ* GHG measurements.

*Table GHG.1. Sampling and analytical procedure for in-situ measurement of GHG fluxes and concentrations*

<table>
<tr><td>*In-situ* GHG fluxes<br>($F_{CO2}$, $F_{CH4}$, $F_{N2O}$)</td><td>Contact persons:<br>Camille Minaudo (University of Barcelona) camille.minaudo@ub.edu<br>Daniel von Schiller (UB) d.vonschiller@ub.edu<br>Biel Obrador (UB) obrador@ub.edu</td></tr>
<tr><td colspan="2">In the field:<br>• Sample air with a syringe, and transfer 20 mL to a 12-mL pre-evacuated gas-tight glass exetainer. Do three replicates at different moments and sites within the subsite. Avoid direct breathing when taking sample.<br>• For each set of chamber measurements, record air temperature and incoming solar radiation inside the chamber, atmospheric pressure.<br>• For each chamber measurement, note the exact time and concentrations if CO2 and CH4 at the start and end of the incubation. Use the time provided by the GHG analyser and note the time zone. For simplicity, it is recommended to always use UTC.<br>• Give an ID to each measurement following this nomenclature: SiteID – Strata (vegetated V/open water OW/bare B) – Sampling Plot (number) – Dark(D)/Light(L) – Date</td></tr>
</table>

(YYYYMMDD). For instance, CUR3-V-9-L-20231025, is a correct GHG chamber measurement ID.

- Start/End time and geographic coordinates of each sampling point will be consigned in pre-prepared field forms.
- If sampling **in open water** (more than 10 cm water depth, no emergent vegetation, regardless of submerged vegetation):
  - Identify and note the main physical characteristics of the measurement: water depth, presence of submerged vegetation, meteorological conditions.
  - Take one water sample following the headspace method (Striegl et al. 2012): 30 mL of water will be collected with a 60 mL polypropylene syringe, creating a headspace with ambient air of 1:1 ratio (sampled water:ambient air). To facilitate the kinetics of equilibration between the liquid and the gas phase, the syringe will be shaken for 1 min and submerged in the water of the sampling site for 30 min to maintain constant equilibrium temperature. Water temperature nearby the syringes during equilibration must be monitored and recorded with a portable sensor. Subsequently, 20 mL of the equilibrated gas will be transferred to a pre-evacuated gas-tight glass exetainer.
  - Deploy the floating chamber (with dark cover) for 10-15 min to catch both diffusive and ebullitive fluxes).
  - At the end of the deployment, sample the air trapped in the chamber with a syringe and transfer 20 mL into a 12-mL pre-evacuated gas-tight glass exetainer.
- If sampling **in vegetated areas** (emergent or submerged with less than 10 cm water depth):
  - Measure with and without the dark cover, for 3-5 min in each configuration.
  - If sampling in a terrestrial plot, the chamber must be sealed to the ground, either by inserting 1 cm into the sediment or by sealing the chamber to the ground using an inert and ductile material, preferably clay (i.e., as used for pottery). Tests have shown that wet sediment from the site as sealing material might produce artefacts (Lesmeister and Koschorreck, 2017). Ensure that everything is airtight by exhaling around the edge of the chamber and at tubing junctions and monitoring the $CO_2$ measurements to make sure there is no spike in numbers before recording the "start" time and concentration.
  - Light intensity measurements should be taken during the clear chamber incubation. Take care to avoid casting a shadow on either the light chamber or the light meter while measurements are taking place. If possible, minimize the occurrence of large changes to the light climate occurring during chamber measurements (e.g., a thick cloud passing overhead halfway through a chamber flux measurement). Take notes of such occurrences if they do happen.
  - At the end of the incubation, sample the air trapped in the chamber with a syringe and transfer 20 mL into a 12-mL pre-evacuated gas-tight glass exetainer.
- If sampling in **bare areas** (soil or sediment exposed to the atmosphere, no apparent vegetation):
  - Measure with the dark cover for 3-5 min.
  - The chamber must be sealed to the ground with pottery clay (see above)
  - At the end of the deployment, sample the air trapped in the chamber with a syringe and transfer 20 mL into a 12-mL pre-evacuated gas-tight glass exetainer.

Description of analytical protocol:

$CO_2$, $CH_4$ and $N_2O$ gas concentrations of air and headspace samples will be measured by gas chromatography with an Agilent Tech. 7820A Gas Chromatograph.

References:

| |
|---|
| • Bastviken et al. 2015. https://doi.org/10.5194/bg-12-7013-2015<br>• Lesmeister and Koschorreck, 2017. https://doi.org/10.5194/amt-10-2377-2017<br>• Striegl et al. 2012. https://doi.org/10.1029/2012GB004306<br>• GHG Measurement Guidelines for Freshwater Reservoirs (https://www.hydropower.org/publications/ghg-measurement-guidelines-for-freshwater-reservoirs) |
| Detailed list of material required in the field:<br>• Portable gas analyser<br>• Extra battery for gas analyser<br>• Tubing for connecting gas analyser to chambers<br>• Floating chamber<br>• Soil/sediment chamber<br>• Cover for chambers<br>• 60-mL polypropylene syringes<br>• Three-way stopcocks<br>• Needles<br>• 12-mL pre-evacuated gas-tight glass exetainers<br>• Light sensor<br>• Air temperature sensor<br>• Water temperature sensor<br>• Air pressure sensor<br>• Wind sensor (if no local meteorological station)<br>• Pottery clay for sealing the chamber<br>• Notebook + pencil<br>• Watch<br>• Marker to label vials<br>• Bag + cooler to store exetainer samples |

## 5.3. Sediment core incubations

### a) In the field

At each subsite, 6 sediment cores are extracted by hand using transparent methacrylate tubes of 50-100 cm in length and 4 cm in diameter. A sediment core consists of approximately 15 cm of sediment, and a water column according to the depth at the sampling point. As soon as the sediment core is taken, it is sealed while ensuring that at least 10 cm of air is left in the uppermost side of the core (headspace) to allow for subsequent gas measurements, even if water column depth at sampling point is deeper.

### b) Incubations in the lab

Once transported intact to the laboratory, the headspace inside the core is air-purged to eliminate the accumulated gases. During this procedure, cores are slightly shaken without disturbing the sediment structure to favour the outflow of gas bubbles. A sample of this headspace is taken and measured to establish initial concentration of gases (atmospheric

pressure and temperature should also be registered). Air samples from headspace are collected from inside the tubes using a syringe with a three-way valve through a rubber plug, and 20 mL of air are transferred in 12 mL pre-evacuated gas-tight glass exetainers. Once air-purged and the initial sample is taken, cores are sealed, and the incubation starts.

The incubation time varies according to the emission rates of the wetlands studied, which is usually between 0.5 and 2 days. Incubation time will be adjusted according to $CO_2$ and $CH_4$ fluxes measured *in-situ* with the floating chambers. Sediment cores obtained from low-emitting sites will be incubated for 2 days, whereas cores from sites presenting large emissions will be incubated for a shorter period.

Incubations will be carried out in bioclimatic chambers adjusting temperature and light as close as possible to the conditions found at the sampling point. The concentrations of $CO_2$, $CH_4$ and $N_2O$ in the headspace of each incubated core are measured at the end of incubation. Potential GHG fluxes from all sediment cores are determined by computing the absolute difference between final and initial concentrations over the incubation time.

$CH_4$ can potentially be emitted to the atmosphere by ebullition rather than diffusion only. To assess this potential flux, the sediment core is shaken vigorously to mobilize to the headspace the gas trapped in the sediment. After this manipulation, an additional sample is taken.

The amount of gas present in the tube is calculated considering the headspace volume and applying the ideal gas equation as explained previously for chamber measurements (see section 5.2).

Table GHG.2 below presents in detail the sampling and experiment procedure for the incubation of sediment cores.

*Table GHG.2. Sampling and analytical procedure for sediment core incubation and potential GHG fluxes.*

| **GHG fluxes – sediment core incubations ($potF_{CO2}$, $potF_{CH4}$, $potF_{N2O}$)** | Contact persons:<br>Antonio Picazo (University of Valencia)<br>antonio.picazo-mozo@uv.es |
|---|---|
| In the field:<br>• Describe in a notebook the environmental setting of the sampling point (water column depth, presence of submerged vegetation). Record the exact geographic coordinates.<br>• Extract 6 sediment cores by subsite using transparent methacrylate tubes of 50-100 cm in length and 4 cm in diameter. Give a unique identifier to each core.<br>• Leave at least 10 cm of air in the uppermost side of the core (headspace), even if water column depth is greater. Measure and report depth of sediment for each core.<br>• Transport to the lab vertically with minimum disturbance.<br>In the lab:<br>• Air-purge the headspace<br>• Gently shake the sediment core to favour the outflow of gas bubbles.<br>• Take a sample of the headspace, corresponding to initial concentrations at the start of incubation. | |

- Place the core in a bioclimatic chamber adjusting temperature and light as close as possible to the conditions found at the sampling point. The incubation time varies according to the emission rates of the wetlands studied, which is usually between 0.5 and 2 days.
- Take a headspace sample, corresponding to the final concentrations at the end of incubation.
- Shake vigorously the core to mobilize by ebullition the gas trapped in the sediment and take a final headspace sample.

Description of analytical protocol:

$CO_2$, $CH_4$ and $N_2O$ gas concentrations will be measured by gas chromatography with an Agilent Tech. 7820A GC.

References:

- Bastviken, D. (2009). https://doi.org/10.1016/b978-012370626-3.00117-4
- Bastviken et al. (2002). https://doi.org/10.1021/es010311p
- Camacho et al. (2017). https://doi.org/10.3390/w9090659

Detailed list of material required in the field:

- Methacrylate tubes of 50-100 cm in length and 4 cm in diameter c tubes (6 per subsite).
- Plastic taps to seal the cores. Tap for the top should have a hole sealed with a rubber tap to allow for headspace sampling with a syringe.
- Marker for labelling cores.
- Plastic containers for the transport of sediment cores vertically. Length of the cores will usually range 0.5-1 m.

Detailed list of material required in the lab:

- Bioclimatic chambers with temperature and light control system.
- 60-mL, 10-mL, and 1-mL polypropylene syringes and dedicated needles.
- 12- mL pre-evacuated gas-tight glass exetainers.
- Three-way stopcocks.
- Notebook + pencil.

## 5.4. Ecological indicators and ancillary data

The measurement of the selected ecological indicators involves sampling and analysing water and sediment samples. *In-situ* procedure and subsequent laboratory analyses are detailed in the following sections:

- Section a) details the sample processing of all water samples.
- Section b) details the sample processing of sediment samples.
- Section c) describes the measurements of meteorological variables

### a) Water samples

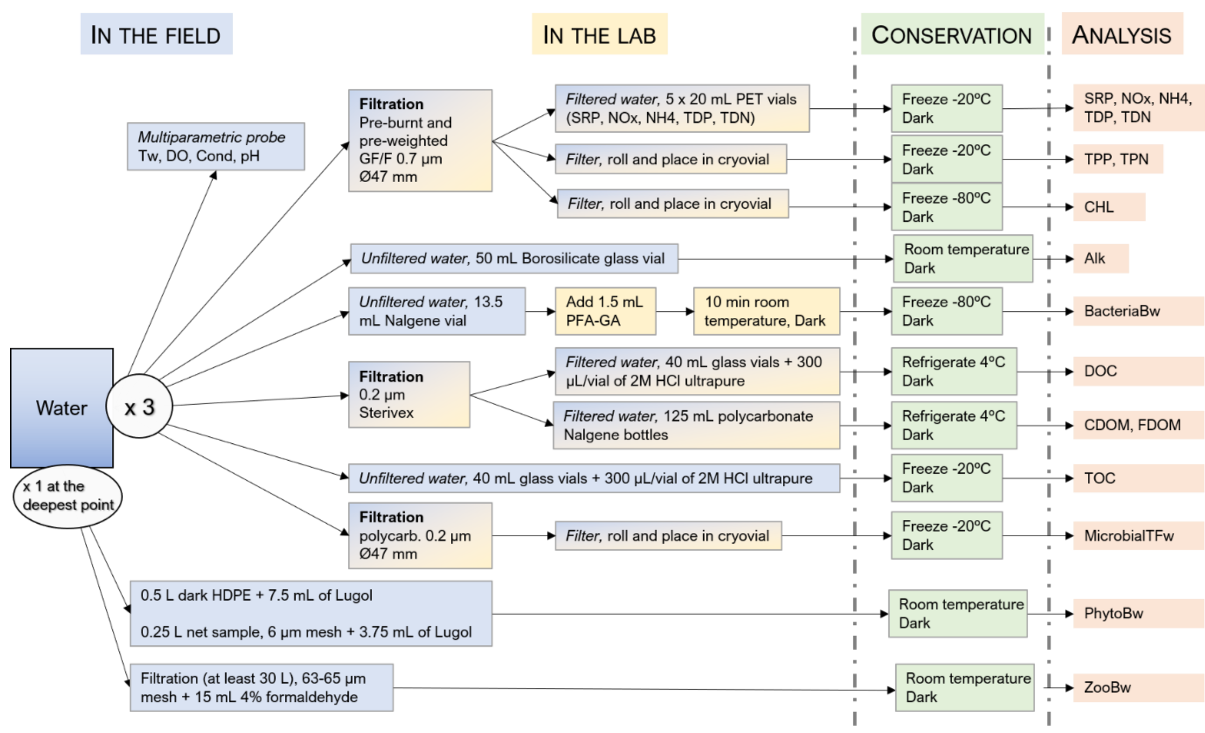


*Figure 5. Overview of the processing steps for sampling at one subsite, from the field (blue boxes) to the lab (yellow boxes) to prepare the water samples for conservation (green boxes) before subsequent lab analyses (red boxes). Blue/yellow boxes indicate steps that will preferably be taken in the field, but can also be taken in the lab.*

At each subsite,

- Select three sampling points within the subsite to cover different spatial configurations and water depth conditions, aiming at maximising sampling representativeness. This leads to triplicate samples at each subsite for most of the ecological indicators.
- Take water samples of 5-10 L at each of the three sampling points. Take samples from a boat if conditions allow. This stock of water sample will then be used for the measurements of all the ancillary data. Always rinse three times the containers and their caps with ambient *in-situ* water before filling them up. Fill up the containers

completely, trying to avoid air inside the containers, as much as possible. Sample the top 10 cm of the water surface.

- measure Tw, DO, Cond and pH with a calibrated multiparametric probe at each sampling point
- report the geographical coordinates of all sampling points (see section 5.1.c)

Additionally, when depth and volume allow, filter directly onsite and at the deepest sampling point:

- 30 L for phytoplankton analyses with a 6-15 µm mesh,
- 30 L for zooplankton analyses with a 63-65 µm mesh.

Place immediately all these samples in a cooler until further processing.

The tables below detail the protocols for sampling and preparation of the samples for conservation until subsequent analyses in the laboratory. These tables are structured as follows:

Table W.1: nutrients

Table W.2: CHL pigments

Table W.3: alkalinity

Table W.4: TOC

Table W5: DOC

Table W.6: CDOM and FDOM

Table W.7: Bacterial abundance (BacteriaBw)

Table W.8: Taxonomic and functional microbial diversity in the water column (MicrobialTFw)

Table W.9: Phytoplankton biomass and composition

Table W.10: Zooplankton biomass and composition

Each of these tables is presented in a similar fashion, including the contact information of the project partner in charge of the analyses.

*Table W.1: Sampling and analytical procedure for nutrient concentrations.*

| Nutrients (TDP, SRP, TPP, TDN, TDP, $NH_4$, NOx = $NO_3$ + $NO_2$) | Contact person:<br><br>Pedro Coelho, Aveiro University (UAveiro)<br><br>jpcoelho@ua.pt |
|---|---|

In the field:

- From a water sample taken, filter at least 300 mL.
- Filtration is done on numbered, pre-combusted (450°C, 5h) Whatman GF/F filter (47 mm diameter, 0.7 ⍰m pore size) under vacuum pressure of less than 0.03 MPa under low light. Gently homogenize the sample water before pouring, by inverting the bottle several times to create a uniform mixture. Volume of filtered sample should be measured with graduated cylinder. The exact volume of filtered water should be recorded.
- Store the filtrate in 3 separate acid washed PET 20 mL vials, corresponding to SRP, $NH_4$, NOx, TDP, TDN.
- Label according to the description in section 5.1 of the manual.
- Keep the filter for TPP and TPN analyses in a 5 mL cryovial.
- Note that filtration can be done in the lab if local conditions don't allow for direct *in-situ* filtration

Conservation

- Freeze at -20°C
- Transfer to project partner where nutrients will be analysed, by courier under dry ice condition, together with a list of sample records over the entire campaign, indicating sample IDs, and volume filtered.

Description of analytical protocol:

Dissolved nutrient (SRP, $NH_4$, NOx, TDN, TDP) quantification will be performed using an automated continuous flow analyser (Skalar San ++), according to Skalar standard methods, and after proceeding with acid treatment for TDN and TDP.

Particulate forms will be analysed by colorimetry after heating and acid digestion for TPP, and elemental analysis for TPN (also refer to Tables Sed.2 and Sed.3 for a description of the analytical protocol).

References:

- Skalar Methods (Catnr. 475-426 issue 042015/MH/99296481, Catnr. 156-002w/r issue 042015/MH/99296481, Catnr. 503-505w/r issue 042015/MH/99296481)
- APHA 4500-N NITROGEN (2018). https://doi.org/10.2105/SMWW.2882.086
- APHA 4500-P PHOSPHORUS (2018). https://doi.org/10.2105/SMWW.2882.093

Detailed list of material required in the field:

- Water sampler
- Acid-washed water container
- Cooler with ice bags
- Filtration system

Detailed list of material required in the lab:

- Whatman GF/F filters, Ø 47 mm, pre-burnt
- Filtration system
- 5 mL and 20 mL PET vials
- Tweezers
- Freezer -20°C

*Table W.2: Sampling and analytical procedure for photosynthetic pigments*

**Photosynthetic pigments (Chlorophylls and taxa specific carotenoids) - CHL**

Contact persons:
Jolita Petkuviene, Klaipeda University (KU)
Jolita.petkuviene@ku.lt
Diana Vaiciute, Klaipeda University (KU)
Diana.vaiciute@jmtc.ku.lt

In the lab:

- Proceed for sampling and filtration as described in Table W.1, wrap filters and place into 5 ml cryogenic vials.
- Volume of filtered sample should be measured with graduated cylinder. The exact volume of filtered water should be recorded.

Conservation:

- Freeze at - 80°C.
- Transfer to project partner where CHL will be analysed, by courier under dry ice condition, together with a list of sample records over the entire campaign, indicating sample IDs, and volume filtered.

Description of analytical protocol:

Photosynthetic pigments are measured using high performance liquid chromatography (HPLC) method following Van Heukelen&Thomas (2001) procedure:

- Glass fibre filters extracted in acetone (90%).
- For extraction samples are placed into the refrigerator for 24 h.
- Samples are disrupted with an ultrasonic bath.
- Extracts are clarified using 0.45-µm, PTFE, HPLC syringe cartridge filters fitted with glass fiber prefilters.
- Analysis with HPLC.

References:

- Van Heukelem and Thomas (2001). https://doi.org/10.1016/S0378-4347(00)00603-4

Detailed list of material required in the field:

- Water sampler
- 1.5 L dark HDPE bottle, acid-washed
- Cooler with ice bags

Detailed list of material required in the lab:

- Whatman GF/F filters, Ø 47 mm
- Filtration system
- 5 mL cryogenic vials
- Tweezers

| • Deep freezer −80°C |
|---|

*Table W.3: Sampling and analytical procedure for alkalinity*

<table>
<tr><td>Alkalinity - Alk</td><td>Contact person:<br>Carlos Rochera (University of Valencia)<br>carlos.rochera@uv.es</td></tr>
<tr><td colspan="2">In the field:<br>• Measure pH in-situ<br>In the field or in the lab:<br>• Transfer a volume of 50 mL of unfiltered water sample into a 100 mL borosilicate glass bottle with screw cap</td></tr>
<tr><td colspan="2">Conservation:<br>Room temperature or fridge 4ºC, in the dark.</td></tr>
<tr><td colspan="2">Description of analytical protocol:<br>Alkalinity of water refers to its acid-neutralizing capacity. This analysis will be performed in the lab by the titration method using a colour pH indicator.<br>Conduct the titration substituting the normality of standard acid solution in the sample for the standard NaOH. The endpoint, which corresponds to the neutralization of acidity present in sample, is determined by the Bromocresol green-methyl red pH indicator solution. This indicator gives a red color in acidic conditions and green color in basic conditions.<br><br>• Add 1-2 drops of Bromocresol green-methyl red pH indicator solution. Sample is expected to turn on blue color as pH is above 7.<br>• After, add at least 5 ml of HCl 0,1 N to the sample with a micropipette. This will produce an excess of acidity which can be further quantified.<br>• Calculate Alkalinity using the following equation:<br><br>$$Alkalinity\left[mg\ \frac{CaCO3}{L}\right] = \frac{(A \times B - C \times D) \times 100}{V}$$<br>Where:<br>A = volume NaOH titrant [mL]<br>B = normality of NaOH<br>C = volume of HCl [mL]<br>D = normality of HCl<br>V = volume of sample [L]</td></tr>
<tr><td colspan="2">References:<br>• APHA Standard Methods. 2320 Alkalinity. https://www.standardmethods.org/doi/10.2105/SMWW.2882.023</td></tr>
<tr><td colspan="2">Detailed list of material required in the field:<br>• pH-meter calibrated the day before sampling<br>Detailed list of material required in the lab:<br>• 100 ml PYREX glass bottle<br>• Micro-pipette<br>• NaOH standard<br>• HCl 0.1N standard</td></tr>
</table>

| • Bromocresol green-methyl red with dropper |
|---|

*Table W.4: Sampling and analytical procedure for TOC concentration*

| Total Organic Carbon (TOC) | Contact person:<br>Chiara Santinelli (CNR)<br>chiara.santinelli@ibf.cnr.it |
|---|---|
| In the field:<br>• Rinse 3 times a 40 mL glass vials with unfiltered water sample.<br>• Fill the 40mL vial with unfiltered water sample.<br>• Dispense 300 µL of 2M high purity HCl in all the vials, close the cap and mix. The HCl and the pipette must be dedicated ONLY to DOC and TOC acidification. | |
| Conservation:<br>Store the samples at -20°C and in the dark. | |
| Description of analytical protocol:<br>Total Organic Carbon concentration will be measured by the Shimadzu Total Organic Carbon analyzer (TOC-L) by high temperature catalytic oxidation following Santinelli et al. (2015, 2021). Samples will be acidified with high purity HCl 2N and sparged for 3 minutes with $CO_2$-free pure air, in order to remove inorganic carbon. Samples (150 µL) will be injected in the furnace (680°C) after rinsing with the sample three times. From 3 to 5 replicate injections will be performed until the analytical precision is lower than 1% (± 1µM). A five-point calibration curve will be done by injecting standard solutions of potassium hydrogen phthalate in the expected concentration range of the samples. At the beginning and end of each analytical day the system blank will be measured using Milli-Q water and the reliability of measurements will be controlled by comparison of data with a TOC reference (CRM) seawater (Hansell, 2005).<br>TOC concentrations will be calculated by the equation:<br>$$TOC\ (\mu M) = \frac{(sample\ area\ -\ system\ blank\ area)}{slope\ of\ standard\ curve}$$<br>This procedure will guarantee the quality of the data and the possibility to compare with previously published estimates in other laboratories. | |
| References:<br>• Hansell (2005). https://doi.org/10.1029/2005EO350003<br>• Santinelli et al. (2015). https://doi.org/10.1016/j.marchem.2015.06.018<br>• Santinelli et al. (2021). https://doi.org/10.5194/bg-2018-418 | |
| Detailed list of material required in the field:<br>• Cooler and icepack storage.<br>• MilliQ water<br>• 40mL vials<br>• Dedicated pipette p1000<br>• Tips for p1000 | |

*Table W.5: Sampling and analytical procedure for DOC concentration*

| **Dissolved Organic Carbon (DOC)** | Contact person:<br>Chiara Santinelli (CNR)<br>chiara.santinelli@ibf.cnr.it |
|---|---|
| In the field:<br>• Refer to the protocol described for TOC, see Table W.4<br>• Wash the Sterivex filter 0.2 µm with 300 mL of sample or, if the suspended matter concentration is high, with 500 mL of MilliQ before use.<br>• Filtrate the water at 0.2 µm through a Sterivex filter.<br>• Rinse 3 times the 40 mL glass vial with filtered samples.<br>• Fill a 40mL vial with filtered water sample.<br>• Dispense 300 µL of 2M high purity HCl in all the vials, close the cap and mix. The HCl and the pipette must be dedicated ONLY to DOC and TOC acidification. | |
| Conservation:<br>Store the samples at 4°C and in the dark. | |
| Description of analytical protocol:<br>The same protocol as for TOC concentration will be used (Table W.4) | |
| References:<br>• Hansell (2005). https://doi.org/10.1029/2005EO350003<br>• Santinelli et al. (2015). https://doi.org/10.1016/j.marchem.2015.06.018<br>• Santinelli et al. (2021). https://doi.org/10.5194/bg-2018-418 | |
| Detailed list of material required in the field:<br>• Cooler and icepacks<br>• MilliQ water<br>• 0.2 µm Sterivex filters<br>• 40 mL vials<br>• Dedicated pipette p1000 + tips | |

*Table W.6: Sampling and analytical procedure for CDOM and FDOM*

| Chromophoric and Fluorescent Dissolved Organic Matter (CDOM and FDOM) | Contact person:<br>Chiara Santinelli<br>chiara.santinelli@ibf.cnr.it |
|---|---|
| In the field:<br>• Refer to the protocol described for TOC, see Table W.4<br>• Proceed to filtration, as described for DOC, see Table W.5.<br>• Rinse 3 times the 125 mL polycarbonate Nalgene bottles with filtered samples.<br>• Fill a 125 mL polycarbonate Nalgene bottles with the filtered sample. | |
| Conservation:<br>Store the samples at 4°C and in the dark. | |
| Description of analytical protocol:<br>**Chromophoric Dissolved Organic Matter (CDOM)**<br>Absorbance spectra of chromophoric DOM (CDOM) will be measured throughout the UV and visible spectral domains (230–700 nm) using a spectrophotometer UV-visible (Shimadzu UV-2600i), with a 10 cm quartz cell following Retelletti Brogi et al., (2022). For each sample 3 spectra will be measured. The absorption spectrum of Milli-Q water will be used as blank and subtracted from each sample | |

spectrum. After the subtraction, the absorption coefficient at 254 nm ($a_{254}$) and the spectral slope between 275 and 295 nm ($S_{275–295}$) will be calculated by using the ASFit software (Omanović et al., 2019). $a_{254}$ can be used to have semi-quantitative information on CDOM, whereas, the $S_{275–295}$ will be calculated in order to obtain indirect information on the average molecular weight and aromaticity of the CDOM pool. In addition, the absorption coefficient at all the wavelengths (between 230 and 600 nm), the indexes and the spectral slopes in different range can be calculated. This data can be also useful for satellite validation of CDOM products.

**Fluorescent Dissolved Organic Matter (FDOM)**
Excitation Emission Matrixes (EEMs) will be obtained by using the Aqualog spectrofluorometer (HORIBA Jobin Yvon) with a 10 x 10-mm quartz cuvette following Bachi et al., (2023). This instrument uses a charge-coupled device to detect the signal, guaranteeing a high acquisition velocity and reduced photobleaching. The characteristics of the lamp improve the sensibility of data acquisition at low excitation wavelengths (250-350 nm) allowing a better identification of the protein-like fluorescence. Emission will be registered between 212 and 620 nm every 3.27 nm (8 pixels) with an integration time of 10 s. Excitation will range between 250 and 450 nm at 5 nm increment. EEMs will be corrected for instrumental bias. Rayleigh and Raman scatter peaks will be removed by using monotone cubic interpolation. The EEMs will be elaborated using the TreatEEM software (Omanovic Dario, TreatEEM-program for treatment of fluorescence EEMs, http://sites.google.com/site/daromasoft/home/treateem). EEMs will be corrected for the inner filter effect using the following equation:

$$F_{corr} = F_{obs} \times 10^{\frac{Abs_{Ex}+Abs_{Em}}{2}}$$

where $F_{corr}$ is the inner filter effect corrected fluorescence intensity, $F_{obs}$ is the measured fluorescence intensity, $Abs_{Ex}$ is the absorbance at fluorescence excitation wavelength, and $Abs_{Em}$ is the absorbance at the selected fluorescence emission wavelength. Absorbance will be measured by the Aqualog spectrofluorometer (HORIBA Jobin Yvon) on the same sample used for fluorescence analysis. The corrected EEMs were normalized dividing the fluorescence intensity by the Raman band of Milli-Q water integrated between 371 and 428 nm ($\lambda_{Ex}$ = 350 nm). Fluorescence will be therefore reported in Raman Units (Lawaetz and Stedmon 2009).
PArallel FACtor (PARAFAC) analysis will be applied to EEMs measured by using the drEEM (decomposition routines for Excitation Emission Matrices) toolbox for MATLAB software (Murphy et al. 2013). The OpenFluor online database, a database of environmental fluorescence spectra, will be used as a validation tool to characterize the validated components (Murphy et al. 2014).

References:


- Hansell. (2005). https://doi.org/10.1029/2005EO350003
- Lawaetz et al. (2009). https://doi.org/10.1366/000370209788964548
- Murphy et al. (2014). https://doi.org/10.1039/c3ay41935e
- Murphy et al. (2013). https://doi.org/10.1039/c3ay41160e
- Omanović et al. (2019). https://doi.org/10.1016/j.cageo.2019.104334
- Retelletti Brogi et al. (2022). https://doi.org/10.1016/J.SCITOTENV.2021.152412
- Bachi et al. (2023). https://doi.org/10.1002/lno.12325


Detailed list of material required in the field:

- Cooler and icepacks
- Freezer for icepacks.
- MilliQ water.

- 125 mL polycarbonate Nalgene bottles

*Table W.7: Sampling and analytical procedure for bacterial abundance in water samples*

| **Bacterial abundance in water samples (BacteriaBw)** | Contact person: Carlos Rochera and Antonio Picazo (University of Valencia) carlos.rochera@uv.es, antonio.picazo-mozo@uv.es |
|---|---|
| In the lab:<br>• Take a homogenised subsample from the unfiltered water stock provided from the sampling of other indicators (e.g., Nutrients, DOC)<br>• Fill up 15 mL centrifuge tube with 13.5 mL of unfiltered water sample from the previous stock<br>• Fix the sample with 1.5 ml (10% of final volume) of a combination of Paraformaldehyde and Glutaraldehyde (PFA-GA) (see below for the preparation of this fixative).<br>• Leave in the dark for 10 min to allow complete cell fixation.<br>• The PFA-GA fixative is prepared as follow:<br>o This protocol is for the preparation of 100 mL of PFA-GA (around 100 samples of a volume of 10 mL can be fixed with this stock)<br>o Weight 10 g of PFA (Paraformaldehyde) (e.g., Sigma P6148).<br>o Place in 88 ml distilled $H_2O$ in a beaker, use a magnetic stirring and a stir bar to dissolve. At this point you can warm the water to facilitate dissolution. Additionally, drops of concentrated NaOH (e.g., 10M) can be added to accelerate notably the dissolution.<br>o After dissolution, adjust the pH to neutrality and add 10 ml PBS (phosphate-buffered saline solution). We use this PBS provided in tablets (e.g., Sigma P4417), in such way that 1 tablet should be diluted in 200 ml of distilled water to reach appropriate concentration.<br>o Add 2 ml Glutaraldehyde 25%<br>o Filter through polycarbonate 0.2 µm in the fume hood<br>o Distribute in 5, 20 ml and 50 ml plastic tubes as desired.<br>o Store the tubes at -20° and use them on demand.<br>o Once unfrozen, a tube should be used within a week or discarded. | |
| Conservation:<br>• Freeze at -80°C if possible, or -20°C if not | |
| Description of analytical protocol:<br>Bacterial abundances are estimated by flow cytometry.<br>• Before running samples in the cytometer, they are stained with SYBR Green-I (Molecular Probes), during 30 min in the dark, to stain double-stranded DNA.<br>• Stained bacterial cells are then visualized by plotting the side scatter light (SSC), which is a proxy of the cell size, versus the green fluorescence (FL1) channel.<br>• The ratios of the green versus red fluorescence emission channels are plotted to discriminate bacteria from the cytometric noise.<br>• Fluorescent beads of 1 µm in diameter are used as size markers. | |

| |
|---|
| • Among all bacteria detected, at least two main subpopulations differing on their green fluorescence intensity are discriminated, proportionally to their DNA content. Accordingly, we refer to these subpopulations as bacteria with a high (HDNA) or low (LDNA) DNA content, respectively, assuming that the HDNA are more active and constitute the dynamic fraction of the assemblage (Lebaron et al., 2001).<br>• The cell concentrations are obtained by considering the volume processed gravimetrically. |
| References:<br>• Rizzo et al. (2017). https://doi.org/10.1016/j.jes.2017.08.007<br>• Lebaron et al (2001). https://doi.org/10.1128/aem.67.4.1775-1782.2001 |
| Detailed list of material required in the field:<br>• 15 mL centrifuge tubes<br>• PFA-GA fixative (see above how to prepare)<br>• Pipette to dispense the fixative<br>• -80°C freezer |

*Table W.8: Sampling and analytical procedure for taxonomic and functional microbial diversity*

| **Taxonomic and functional microbial diversity in the water column (MicrobialTFw)** | Contact person:<br>Carlos Rochera and Antonio Picazo (University of Valencia) carlos.rochera@uv.es, antonio.picazo-mozo@uv.es |
|---|---|
| In the field or in the lab:<br>• Filter as much water as possible (ideally around 3 L) of water sample through a polycarbonate 0.22 µm membrane filter of a diameter of 47 mm. Report the volume filtered.<br>• After filtration, fold the filter on the base of the filtration unit and place it in 1.5 ml Eppendorf tube. | |
| Conservation:<br>• Freeze at -20°C or -80ºC, keep in the dark | |
| Description of analytical protocol:<br>• All the analytical procedures related with the environmental DNA extraction, purification, sequencing and bioinformatic analysis will be performed in the UVEG lab once responsible of pilot sites sent samples to Spain.<br>• DNA extraction will be performed using the EZNA DNA isolation kit (Omega Bio-Tek, Inc., Norcross, GA, United States)<br>• Sequencing of the V4 region of the 16S rDNA gene was performed using the Illumina MiSeq system (2x250 bp)<br>• The Illumina compatible dual indexed amplicon libraries of the16S-V4 rRNA hypervariable region will be created with primers 515f/806r. PCR conditions are extensively detailed in Picazo et al. (2019).<br>• The sequences obtained from the Illumina analyses will be processed by running a pipeline of USEARCH commands with USEARCH v11.0.667 (Edgar, 2013).<br>• The functional organization of the microbial community will be inferred using for instance the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) from the 16S rDNA gene data. The use and principles of this bioinformatic tool is widely described by the developers (Douglas et al. 2020). | |

| |
|---|
| • Functional inferences of the prokaryotic community were performed by screening the available annotated genes within the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.kegg.jp/) catalogue following Ye and Doak, (2009). |
| References:<br>• Picazo et al. (2019). https://doi.org/10.3389/fmicb.2019.00908<br>• Edgar (2013). https://doi.org/10.1101/081257<br>• Douglas et al. (2020). https://doi.org/10.1038/s41587-020-0548-6<br>• Ye and Doak (2009). https://doi.org/10.1371/journal.pcbi.1000465 |
| Detailed list of material required on the field:<br>• 47 mm diameter filtration system<br>• 47 mm diameter Polycarbonate with 0.22 μm pore size<br>• 1.5 ml Eppendorf tubes to store filters |

*Table W.9: Sampling and analytical procedure for phytoplankton biomass and composition*

| **Phytoplankton biomass and composition (phytoBw)** | Contact persons:<br>Jessica Titocci (CNR-IRET, LifeWatch-ERIC) jessica.titocci@iret.cnr.it<br>Alberto Basset (LifeWatchERIC, UNISAL) alberto.basset@unisalento.it |
|---|---|
| In the field:<br>• Sample 0.5 L with a polyethylene bottle from the subsurface. This will be used for quantitative analysis of phytoplankton abundance and species composition.<br>• Where possible (depth > 1.5 m), proceed with 10 m horizontal tow with a net sample using a 6-15 μm mesh plankton net to investigate the presence of rare or low abundant species and for qualitative analysis. Place the sample in 250 ml polyethylene bottle<br>• Fix both samples with Lugol's solution (15 ml/L of sample). | |
| Conservation:<br>Fixed samples will be stored at room temperature and in the dark. To avoid problems due to the preservation, samples will be examined in a short time after sampling (within few weeks). | |
| Description of analytical protocol:<br>Phytoplankton taxonomic identification, the estimation of cell densities and morphometric measurements will be performed using a Nikon T300E inverted microscope (Nikon T300E, Nikon Eclipse Ti) connected with a video-interactive image analysis system, following the Utermöhl method (1958) at 400x magnification. For each sample, at least 400 organisms will be counted and identified to the lowest taxonomic level possible. The cell densities detected will be referred to a liter of water (cell/L). Cell biovolumes (expressed in $\mu m^3$), surface area (expressed in $\mu m^2$) and surface area to volume ratio (S/V) will be estimated according to the species/taxa specific shape association and using the geometric equations recorded on the phytoplankton "Atlas of Shapes" (https://www.phytovre.lifewatchitaly.eu/vre/shapes-groups/). The geometric shape will be attributed to the shape of the individual cell, even in coenobial, colonial and filamentous species where cells are not observable. Cellular carbon content of phytoplankton organisms (expressed as μg C/cell) will be obtained indirectly by converting cell biovolume into carbon using empirically or theoretically derived equations (Menden-Deuer and Lessard, 2000, doi: 10.4319/lo.2000.45.3.0569). The total biovolume of phytoplankton organisms per unit volume of the water ($\mu m^3$/L) will be estimated by multiplying the average biovolume of each taxon identified by its corresponding | |

| density. The total carbon content of phytoplankton organisms (µg C/L) will be calculated by multiplying the mean intracellular carbon value of each taxon by its respective density.<br>Phytoplankton data will be collected and harmonized according the "Phytoplankton data template" (https://www.phytovre.lifewatchitaly.eu/phyto-data-template/) and taxonomic validation will be carried out using WORMS World Register of Marine Species (https://www.marinespecies.org/) and Algae Base (https://www.algaebase.org/). |
|---|
| References:<br>• "Atlas of Shapes". https://www.phytovre.lifewatchitaly.eu/vre/shapes-groups/<br>• Menden-Deuer and Lessard (2000). https://doi.org/10.4319/lo.2000.45.3.0569<br>• "Phytoplankton data template". https://www.phytovre.lifewatchitaly.eu/phyto-data-template/ |
| Detailed list of material required in the field:<br>• Plankton net (25 cm diameter, 6-15 µm mesh)<br>• Dark polyethylene bottles (250 ml, 500 ml)<br>• Lugol |

*Table W.10: Sampling and analytical procedure for zooplankton biomass and composition*

| **Zooplankton biomass and composition (zooBw)** | Contact persons:<br>Anis Guelmami (Tour du Valat) guelmami@tourduvalat.org<br>Samuel Hilaire (Tour du Valat) hilaire@tourduvalat.org |
|---|---|
| In the field:<br>• At the sampling point, filter 30 L (if possible) of water through a plankton net that usually has a 63-65 µm opening. In standing waters, to collect the zooplankton organisms, if possible use a 10 L transparent plankton trap (ex. Schindler-Patalas).<br>• Transfer the filtered content to a 150 mL sampling bottle by rinsing the net using MilliQ and a wash bottle.<br>• On the label and the notebook, specify the volume filtered.<br>• Preserve the sample sing 15 mL 4% buffered formaldehyde solution (probably to be replaced by ethanol).<br>• Place in a cooler and in the dark until 4°C refrigeration in the lab | |
| Conservation:<br>• 4°C, in the dark, and after fixation with formaldehyde (probably to be replaced by ethanol). | |
| Description of analytical protocol:<br>The "EPA Standard Operating Procedure for Zooplankton Analysis" will be used as the main baseline for the analyses of zooplankton samples. | |
| References:<br>• Goswami, S.C. (2004). https://drs.nio.org/drs/handle/2264/95?show=full<br>• EPA Standard Operating Procedure for Zooplankton Sample Collection and Preservation and Secchi Depth Measurement Field Procedures (2017). https://www.epa.gov/sites/default/files/2017-01/documents/sop-for-zooplankton-sample-collection-preservation-and-secchi-depth-measurement-field-procedures-201303-7pp.pdf | |

- EPA Standard Operating Procedure for Zooplankton Analysis (2017). https://www.epa.gov/sites/default/files/2017-01/documents/sop-for-zooplankton-analysis-201607-22pp.pdf

Detailed list of material required in the field:

- Plankton net, 63 -65µm pore size, 0.5-m diameter
- Net sample bucket with a 61-µm pore size metal screen
- PVC frame hand sieve (50-65µm)
- Plastic sample bottles (150mL)
- Wash bottle + MilliQ water

Detailed list of material and reagent required for the analyses:

- Dissecting microscope with 10x to 50x magnification
- Compound microscope with 100x to 1000x magnification
- 1 mL Calibrated Hensen-Stempel pipette
- 100 mL, 250mL and 500mL graduated cylinders
- Ward counting wheel or other suitable counting chamber
- Sedgwick-Rafter counting cell
- Cover glass for Sedgwick-Rafter counting cell
- Microscope slides 1 x 3 inch
- Cover slips
- Small sieves with 63µm and 500µm mesh
- Microprobe
- Micro-forceps
- Formalin (37% formaldehyde solution)
- Ethanol
- 5% Sodium hypochlorite solution (Clorox bleach)
- Rose Bengal stain dissolved in ethanol
- Dilute solution of laboratory detergent

## b) Sediment samples

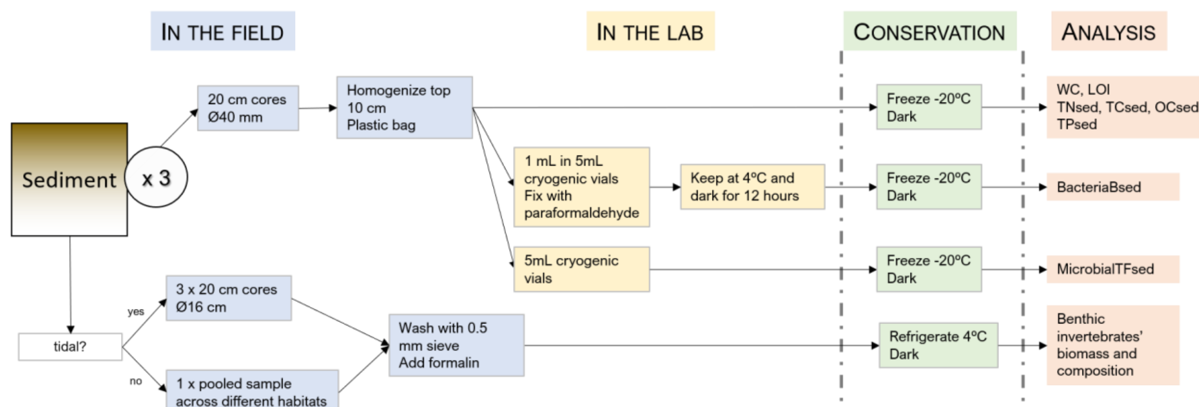


*Figure 6. Overview of the processing steps at one subsite, from the field (blue boxes) to the lab (yellow boxes) to prepare the sediment samples for conservation (green boxes) before subsequent lab analyse (red boxes).*

At each 3 sampling points where water samples were taken (see section b), take a 20 cm long sediment core by hand with Ø 4 cm liners to characterize sediment physical and biogeochemical properties, bacterial abundance, and taxonomic and functional benthic microbial diversity. Use gloves and make sure not to contaminate the samples.

For benthic invertebrates' biomass and composition, different strategies will be employed, depending on the subsite characteristics:

- In tidal environments, 3 short cores with Ø 16 cm will be taken by hand. In deep zones, grabs will replace the cores (3 as well)
- Otherwise, in (shallow) fordable place, a single sample pooled over different habitats will be collected with a 250 µm handnet.

In both cases and in a subsequent step, samples are washed in situ with a 0.5 mm sieve. Note that samples for benthic invertebrates' biomass and composition will only be taken twice, in winter (January-February 2024) and spring (April-May 2024).

The tables below detail the protocols for sampling, preparation of the samples for conservation until subsequent analyses in the laboratory. These tables are structured as follows:

Table Sed.1: Water content (WC) and organic matter derived from loss on ignition (OMsed)

Table Sed.2: Total carbon (TCsed), Organic carbon (OCsed), total nitrogen (TNsed) contents

Table Sed.3: Total phosphorus content (TPsed)

Table Sed.4: Bacterial abundance (BacteriaBsed)

Tables Sed.5: Taxonomic and functional benthic microbial diversity (MicrobialTFsed)

Table Sed.6: Benthic invertebrates, biomass and composition

Each of these tables is presented in a similar fashion, including the name, affiliation and email address of the project partner in charge of the analyses.

*Table Sed.1: Sampling and analytical procedure for sediment water content and organic matter content*

| **Sediment water content and loss on ignition (WC and OMsed)** | Contact persons:<br>Katrin Attermeyer, WasserCluster Lunz (WCL)<br>katrin.attermeyer@wcl.ac.at<br>Benjamin Misteli (WCL) Benjamin.Misteli@wcl.ac.at |
|---|---|
| In the field:<br>• Collect 3 sediment cores per subsite by hand.<br>• Take depth-integrated sediment sample from the upper 10 cm from each core.<br>• Homogenize the upper 10 cm sample and transfer into plastic bag. | |
| Conservation:<br>• Store in –20°C freezer | |
| Description of analytical protocol:<br>Sediment water content and organic matter content will be estimated by weight loss measurements in core sub-samples subjected to sequential heating following standard procedures (Schumacher 2002):<br>1. Weigh aluminium bowl empty.<br>2. Place moist sediment sample in the bowl and weigh combined mass.<br>3. Dry the sample in the oven at 105°C until constant weight (may take up to a 1-2 days).<br>4. Place in desiccator until cooled down and re-weigh the bowl+subsample -> difference yields the water content (WC).<br>5. Set the muffle furnace temperature to 450°C and muffle sample for 4 hours.<br>6. Remove bowl+sample from furnace and place in desiccator until cooled down and re-weigh bowl+sample -> difference from the dry state yields the organic content (OMsed). | |
| References:<br>• Schumacher, B A. (2002). https://cfpub.epa.gov/si/si_public_record_report.cfm?Lab=NERL&dirEntryId=63371 | |
| Detailed list of material required in the field:<br>• Liners, liner tube cutter and slicer (for sampling upper 10 cm)<br>• Containers for sediment (plastic bag)<br>• Cooler with ice packs | |

*Table Sed.2: Sampling and analytical procedure for TCsed, OCsed, and TNsed and C-N stable isotope signatures*

| **Sediment carbon (TCsed), organic carbon (OCsed), and nitrogen (TNsed) and their stable isotope signatures** | Contact persons:<br>Katrin Attermeyer, WasserCluster Lunz (WCL)<br>katrin.attermeyer@wcl.ac.at<br>Benjamin Misteli (WCL) Benjamin.Misteli@wcl.ac.at |
|---|---|
| In the field, in the lab:<br>• See description for WC and LOI above (Table Sed.2).<br>• A subsample from dried sediments obtained after proceeding with WC and LOI (see Table Sed.2) will be used for these analyses | |

| Description of analytical protocol:<br>Sediment total carbon (TCsed), organic carbon (OCsed) and nitrogen (TNsed) content as well as their stable isotope signatures will be analyzed using elemental analysis following standard procedures (Zimmermann et al. 1997):<br>1. Weigh subsample of dried sediments (~1-5 mg) in one tin and one silver capsule.<br>2. Acidify subsample in silver capsule with 37% (w/w) HCl for removal of carbonates, dry, and weigh again<br>3. Analyze TC, OC, N on a Flash HT Plus CNSOH elemental analyzer (Thermo Fisher Scientific, Bremen, Germany) coupled to an isotope ratio mass spectrometer (IRMS) for analyses of stable isotope ratios of sediment TC, OC and N. |
|---|
| References:<br>• Zimmermann et al. (1997). https://cfpub.epa.gov/si/si_public_record_report.cfm?Lab=NERL&dirEntryId=309418 |

*Table Sed.3: Sampling and analytical procedure for total phosphorus content in the sediment, TPsed*

| **Phosphorus content in the sediment (TPsed)** | Contact person:<br>Constantin Cazacu (University of Bucharest, UNIBUC)<br>constantin.cazacu@g.unibuc.ro |
|---|---|
| In the field, in the lab:<br>• See description for WC and LOI above (Table Sed.2).<br>• A subsample from dried sediments obtained after proceeding with WC and LOI (see Table Sed.2) will be used for these analyses | |
| Description of analytical protocol:<br>The determination of TPsed relies on the ignition method:<br>• Weigh 0.5 g of dried and ground sediment sample into a crucible.<br>• Ignite the sample in a muffle furnace at 550°C for 4 hours.<br>• Cool the crucible in a desiccator.<br>• Add 5 mL of 1 N HCl to the crucible and boil for 10 minutes.<br>• Cool the crucible and transfer the solution to a 50 mL volumetric flask.<br>• Dilute to volume with distilled water.<br>• Filter through Whatman No. 42 filter paper.<br>• Analyze the filtrate for orthophosphate using standard colorimetric methods (molybdenum blue).<br>• Absorbance of the resulting molybdenum blue solution will be measured using a UV/Vis spectrophotometer at 885 nm. | |
| References:<br>• Andersen et al., 1976. https://doi.org/10.1016/0043-1354(76)90175-5. | |

*Table Sed.4: Sampling and analytical procedure for bacterial abundances in the sediment (BacteriaBsed)*

<table>
<tr><td>Bacterial abundances in sediment samples (BacteriaBsed)</td><td><u>Contact person:</u><br>Carlos Rochera and Antonio Picazo (University of Valencia) carlos.rochera@uv.es, antonio.picazo-mozo@uv.es</td></tr>
<tr><td colspan="2"><u>In the lab:</u><br>• Take a homogenised subsample from the sediment stock provided from the sampling of sediment WC (see Table Sed.1). For the manipulation of samples, use a spoon previously cleaned with sterilizing alcohol.<br>• Fix 1 ml of sediment with 3 ml of PBS buffered paraformaldehyde (~40% stock solution). It is desirable to use commercial formaldehyde solution stabilized with methanol, which prevents precipitation of polymers.<br>• Keep the fixative acting at 4°C overnight, in the dark.</td></tr>
<tr><td colspan="2"><u>Conservation:</u><br>• Freeze at -20° C until analysis</td></tr>
<tr><td colspan="2"><u>Description of analytical protocol:</u><br>• This method involves the addition of the dispersant tetrasodium pyrophosphate (PPi) to fixed samples and the subsequent application of ultrasound for physical separation of the bacterial cells.<br>• Dilute a volume of fixed-sediment sample with 1 ml of 0.01 M PPi and disperse it with a sonicator.<br>• Proceed as described for the flow cytometry analysis of water samples (see Table W.7).</td></tr>
<tr><td colspan="2"><u>References:</u><br>• Sunamura et al. 2003 (https://doi.org/10.1016/s0167-7012(02)00224-5)<br>• Kemp et al (eds.) 1993 (https://doi.org/10.1201/9780203752746)<br>• Rizzo et al. (2017). https://doi.org/10.1016/j.jes.2017.08.007<br>• Lebaron et al (2001). https://doi.org/10.1128/aem.67.4.1775-1782.2001</td></tr>
<tr><td colspan="2"><u>Detailed list of material required in the lab to preserve samples:</u><br>• 5 ml sterile cryogenic vials<br>• 100-200 ml sterile plastic containers to homogenise samples<br>• Spoons for the manipulation of samples (different sizes will be needed to both homogenise the stock sample and to subsequently fill up the cryogenic vials)<br>• 70-75% ethanol for cleaning purposes<br>• PBS-Buffered paraformaldehyde (~40% stock solution). It is desirable to use commercial formaldehyde solution stabilized with methanol, which prevents precipitation of polymers.<br>• Refrigerator (4°C) and freezer (-20°C)<br>• Dedicated micropipette and tips</td></tr>
</table>

*Table Sed.5: Sampling and analytical procedure for taxonomic and functional microbial diversity in sediment samples (MicrobialTFsed)*

| Taxonomic and functional microbial diversity in sediment samples (MicrobialTFsed) | Contact person:<br>Carlos Rochera and Antonio Picazo (University of Valencia) carlos.rochera@uv.es, antonio.picazo-mozo@uv.es |
|---|---|

In the lab:

- Take a homogenised subsample from the sediment stock provided from the sampling of sediment WC (see Table Sed.1). For the manipulation of samples, use a spoon previously cleaned with sterilizing alcohol.
- Fill up a 5 mL sterile cryovial (leave a headspace to prevent overflow).

Conservation:

- Keep frozen (at least -20ºC) until the day of DNA extraction

Description of analytical protocol:

- All the analytical procedures related with the environmental DNA extraction, purification, sequencing and bioinformatic analysis will be performed in the UVEG lab once responsible of pilot sites sent samples to Spain.
- DNA extraction will be performed using the EZNA DNA isolation kit (Omega Bio-Tek, Inc., Norcross, GA, United States)
- Sequencing of the V4 region of the 16S rDNA gene was performed using the Illumina MiSeq system (2x250 bp)
- The Illumina compatible dual indexed amplicon libraries of the16S-V4 rRNA hypervariable region will be created with primers 515f/806r. PCR conditions are extensively detailed in Picazo et al. (2019).
- The sequences obtained from the Illumina analyses will be processed by running a pipeline of USEARCH commands with USEARCH v11.0.667 (Edgar, 2013).
- The functional organization of the microbial community will be inferred using for instance the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) from the 16S rDNA gene data. The use and principles of this bioinformatic tool is widely described by the developers (Douglas et al. 2020).
- Functional inferences of the prokaryotic community were performed by screening the available annotated genes within the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.kegg.jp/) catalogue following Ye and Doak, (2009).

References:


- Picazo et al. (2019). https://doi.org/10.3389/fmicb.2019.00908
- Edgar (2013). https://doi.org/10.1101/081257
- Douglas et al. (2020). https://doi.org/10.1038/s41587-020-0548-6
- Ye and Doak, (2009). https://doi.org/10.1371/journal.pcbi.1000465


Detailed list of material required in the lab:

- 5 ml sterile cryogenic vials
- 100-200 ml sterile plastic containers to homogenise samples
- Spoons for the manipulation of samples (different sizes will be needed to both homogenise the stock sample and the subsequent fill up the cryogenic vials)
- 70-75% ethanol for cleaning purposes
- Refrigerator (4°C) and freezer (-20°C)
- Dedicated micropipette and tips

*Table Sed.6: Sampling and analytical procedure for benthic invertebrates' biomass and composition (Benthic Invertebrates)*

<table>
<tr><td>Benthic macroinvertebrates' biomass and composition</td><td><u>Contact persons:</u><br>Heliana Teixeira (University of Aveiro) heliana.teixeira@ua.pt<br>Anis Guelmami (Tour du Valat) guelmami@tourduvalat.org<br>Samuel Hilaire (Tour du Valat) hilaire@tourduvalat.org</td></tr>
<tr><td colspan="2"><u>Note:</u><br>Samples for benthic invertebrates' biomass and composition will only be taken twice, during the winter campaign (January 2024) and summer campaign (July 2024).<br><u>Description of sampling protocol:</u><br>• In intertidal sites, take by hand 3 sediment cores of 15 to 25 cm depth and Ø 16 cm. Note the exact depth of sediment sampled. In deep zones, grabs will replace the cores (3 as well)<br>• Otherwise, in (shallow) fordable place, a single sample pooled over different habitats will be collected with a 250 µm handnet.<br>• Wash sample with 0.5 mm sieve.<br>• Fix with 70 % ethanol.</td></tr>
<tr><td colspan="2"><u>Conservation:</u><br>• Keep refrigerated, in the dark.</td></tr>
<tr><td colspan="2"><u>Description of analytical protocol:</u><br>• In the laboratory, samples are washed and sieved through sieves of gradual mesh sizes as deemed necessary (5 mm, 2 mm, 1 mm).<br>• Each fraction is stored in ethanol and refrigerated until taxonomic identification can be carried out.<br>• Taxonomic identification and counting of individuals are done at the species level or to the lowest reliable taxonomic level. The WoRMS - World Register of Marine Species should be followed as reference taxonomic database (https://www.marinespecies.org).<br>• The ash free dry weight (AFDW) of individuals in a sample is assessed after combustion for 8 h at 450ºC, for the determination of individual samples biomass (W).</td></tr>
<tr><td colspan="2"><u>References:</u><br>• Chainho et al. (2007). https://doi.org/10.1016/j.marpolbul.2007.06.009<br>• Muxika et al. (2007). https://doi.org/10.1016/j.marpolbul.2006.05.025<br>• Karakassis et al. (2013). https://doi.org/10.1016/j.ecolind.2012.12.020</td></tr>
<tr><td colspan="2"><u>Detailed list of material required on the field:</u><br>• (intertidal) Ø 16 cm sediment corer (equivalent to 0.02 $m^2$)<br>• Handnet (250 µm mesh)<br>• 0.5 mm sieve (alternatively 0.5 mm mesh bag)<br>• 70 % ethanol<br>• Containers + plastic bags for individual samples</td></tr>
</table>

### *c) Meteorological conditions*

Meteorological conditions during sampling represent crucial data to constrain air/water GHG exchange coefficients, and to enable the comparison of GHG fluxes measurements obtained under variable meteorological conditions. We will rely on nearby meteorological stations for wind speed, incoming radiation (SWR), air temperature (Tair), atmospheric pressure (Patm).

This data will be combined with high-frequency *in-situ* measurements of SWR, Tair, Patm using HOBO U20 pressure sensor and HOBO Pendant MX Temperature/Light sensors, or equivalent. The corresponding sensors will be deployed together upon arrival at the subsite and left to record every 10 seconds, making sure not to shield the sensors while working nearby, until the fieldwork team is ready to move to another subsite.

# 6. Refinement of the LUPLES method

To quantify the main pressures affecting lentic ecosystems, a method based on land uses in the catchment area will be implemented after improvement (Morant et al, 2021). This method, LUPLES (Land Uses for estimating Pressure Levels to approach the Ecological Status), is based on the quantification of the relative influence of land uses on the four main pressures related to pollution experienced by lentic ecosystems, namely: eutrophication, organic enrichment, acidification, and pollutants pressures. The land uses are analyzed by using the delineation and classification according to the European Corine-Land Cover (CLC) (EEA 2019). Land uses are aggregated into groups from the different land cover uses that appear disaggregated at the third level category of CLC:

- Irrigated agriculture areas, like rice fields, vegetables and fruit trees (CLC codes 212, 213 and 222).
- Rain-fed agriculture, including vineyards, olive groves, annual crops associated with permanent crops, mosaic crops, agricultural lands with significant natural and semi-natural vegetation spaces, as well as agroforestry systems (CLC codes 211, 221, 223, 241, 242, 243, 244).
- Pressures exerted by livestock, estimated from land uses like meadows and natural pastures, (CLC codes 231 and 321).
- Urban uses by continuous and discontinuous urban structure, industrial and commercial areas, railways and associated lands for these networks, port areas, airports, areas under construction, green urban areas, and sports and leisure facilities, which correspond to the CLC codes 111, 112, 121, 123, 124, 133, 141, 142, respectively.
- Finally, communication infrastructures (code 122), dumps (code 131), and mining areas (code 132) were also mapped and assessed, each separately.
- Natural uses are not considered in the LUPLES index for the pressures assessment due to their low impact regarding the pressures identified in comparison with the other land uses promoted by the human activities.

To normalize the data for their use as a pressure estimation, the percentage of the area occupied by each of these land use groups regarding the total catchment is used, as follows:

$$Land\ use\ pressure\ level = \frac{land\ use\ group\ area\ [km^2]}{total\ catchment\ area\ [km^2]} \cdot 100$$

Once normalized, the relative contribution of each land use type to the level of each specific pressure related to pollution (eutrophication, organic enrichment, acidification and specific pollutants) is obtained by using a weighting factor, according to the relative influence of each land use on each pressure type. The weighting factor is assigned by ranking each land use over a range from 0 to 1, as low (0.0 to 0.2), middle (0.3 to 0.6) and high (0.7 to 1). The determination

of the degree of contribution (specific weighting factor) of each land use to each pressure type is fixed according to Morant et al. (2021).

To obtain the final pressure value, the percentage of each relative area of the different land use groups in the catchment is multiplied by their weighting factor for the pressure type evaluated (eutrophication, organic enrichment, acidification and specific pollutants), then these weighted relative contributions are added to sum up the pressure level of each waterbody for this specific pressure type. Table 6.1 summarizes the weighting factors used for the estimation of the contribution of each land use class to the different pressure type.

***Table 6.1.*** *Weighting factors applied for the quantification of the pressure type level for each land use group.*

| Land use group | Eutrophication | Organic enrichment | Acidification | Specific Pollutants |
|---|---|---|---|---|
| Irrigated agriculture | 0.8 | 0.2 | 0.0 | 0.7 |
| Rainfed agriculture | 0.3 | 0.1 | 0.0 | 0.3 |
| Pastures (livestock) | 0.3 | 0.4 | 0.3 | 0.5 |
| Urban uses | 0.4 | 0.6 | 0.2 | 0.5 |
| Communication infrastructures | 0.0 | 0.0 | 0.2 | 0.7 |
| Mining | 0.0 | 0.0 | 0.9 | 0.9 |
| Dumps | 0.7 | 0.8 | 0.8 | 0.8 |

This index will be reviewed, and the algorithms coefficients will be improved with the data obtained in the RESTORE4C project (both case pilots and the meta-analysis.

## References

European Environmental Agency. 2019. CORINE Land Cover; European Environmental Agency: Copenhagen, Denmark, 2019. Available online: https://land.copernicus.eu/pan-european/corine-land-cover

Morant D, Perennou C, Camacho A. 2021. Assessment of the pressure level over lentic waterbodies through the estimation of land uses in the catchment and hydro-morphological Alterations: The LUPLES method. Applied Sciences, 11(4), 1633. https://doi.org/10.3390/app11041633