



Deliverable 1.2  
**Standard Operational Procedures**  
*March 2023*

## 1. Introduction

This document provides the detailed sampling, preservation and analysis of samples protocols defined by the ARENA Consortium for the analysis of the different parameters considered within this project. The present document is intended to be a basis for laboratory and data analysis, but with the possibility of modifications based on needs arising along the duration of the project.

## 2. Protocols

### 2.1 Sediments sampling for microbiome analysis

Aliquots of surface sediments (0–1 cm), in the order of grams, will be collected from cores by scuba divers or by Van Venn grabs and using sterile falcon tube (50 ml) and immediately stored at -20°C or in boxes with ice packs until shipping and/or laboratory analysis.

### 2.2 Water sampling for microbiome analysis

One liter of surface seawater, in duplicate, will be collected in sterilized containers and kept in the dark until freezing or further processing. A vacuum filtration system or a peristaltic pump will be used for the filtration of seawater 0.22 µm pore size sterile membrane filters (diam. 47 mm). Filters will be stored at -20°C until further processing.

### 2.3 Fish feed sampling for microbiome analysis

Approximately 5-10 g of feed samples will be collected in sterile tubes and preserved at -20°C until further processing.

### 2.4 Fish tissues sampling for microbiome analysis

Adult individuals of fish (sea bream and sea bass) will be collected by a fishing net, immediately euthanized with a lethal dose (0.5 g/L) of Tricaine methanesulfonate (MS222), according to the ethical principles and national legislative context, and placed on ice until arrival at the laboratory around 1 h later; weight, total, and standard length will be measured. Fish specimens will be dissected within 4 h of capture. For each fish specimen, we will isolate: (1) fish skin (a 2 × 2 cm square from the left side), (2) fish gill (second gill arch on the left gill), (3) fish gut (fore-, mid-, and hindgut separated; feces or digesta where possible), (4) fish fillet (2 × 2 cm square at full depth from the left side). Except for fish feces, all the other tissue samples will be rinsed with sterile phosphate buffer solution to remove possible loosely attached microorganisms. All samples will be obtained by dissecting fish specimens using sterile scalpels and scissors, immediately placed in sterile tubes, and stored at – 20 °C until further analyses.

### 2.5 DNA extraction sediments, seawater, fish tissues, and feed samples

DNeasy PowerSoil kit (QIAGEN) will be used following the manufacturer's recommendation and with some slight modifications as reported in Quero et al. (2022). DNA's quality and quantity will be assessed using spectrophotometric or fluorometric measurements (e.g., Nanodrop or Qubit).

### 2.6 Microbiome and resistome analysis by shotgun metagenomics

Shotgun metagenomics will be performed by external services on indexed DNA libraries with approximate fragment length of 500 bp. Library sequencing will be performed using Illumina NextSeq instruments, with NexSeq High or Mid Output Illumina sequencing kits, in order to obtain 2x150 pair end reads (300 cycles) and >3 Gb per sample. The obtained data will be processed thanks to bioinformatic pipelines to characterize the microbial community composition and the antibiotic resistome (total content of antibiotic resistance genes (ARGs)). In detail, the reads will be assessed using FastQC (version 0.11.9) (Andrews, 2010) and MultiQC (version 1.12) (Ewels et al., 2016). After the quality check the illumina adapters will be removed using Trimmomatic (version 0.39) (Bolger et al., 2014) and the paired reads will be merged using vsearch (version 2.17.1) (Rognes et al., 2016). The taxonomic profiling of the reads will be done using different pipelines to obtain the best output (e.g., Metaphlan3, (Beghini et al., 2021)). The bacterial abundances will be expressed in a relative manner as percentages. ARG-like sequences will be annotated with DeepARG database (deepARG-DB-v1.1.1) (Arango-Argoty et al., 2018). Copies of 16S rRNA genes will be inferred by mapping against the 2013 release of the GREENGENES database (DeSantis et al., 2006). The abundances of ARGs will be normalized using the copy number of the 16S rRNA gene per each sample. Furthermore, the metagenome assembled genomes (MAGs) will be recovered after assembly or co-assembly the cleaned reads with MEGAHIT version 1.2.9 (Li et al., 2015) following a previously published method (Parks et al., 2017). The bins will be refined and only MAGs with high quality score will be taxonomically assigned with GTDB-Tk version 2.1.0 (Chaumeil et al., 2020). To identify the MAGs containing ARGs, predicted coding sequences (pCDS) for each MAG will be generated using prodigal version 2.6.3 (Hyatt et al., 2010) and ARG-like sequences will be annotated using the DeepARG pipeline for long sequences (--model LS) against the DeepARG database (deepARG-DB-v1.1.1) (Arango-Argoty et al., 2018).

## 2.7 qPCR

The quantified DNA samples (in a similar concentration) will be tested to quantify selected antibiotic resistance genes (ARGs) and the 16S rRNA gene by quantitative Real Time PCR (qPCR). A standard curve for each selected ARG and for the 16S rRNA gene will be prepared as described in Di Cesare et al. (2013; <https://doi.org/10.1371/journal.pone.0062838>). In detail the selected target will be amplified, purified and ten-fold diluted. The standards, DNA samples and at least four no template controls (NTC) will be tested in the same reaction. For each selected gene the limit of quantification (LOQ), reaction efficiency and R2 will be determined as described in Bustin et al. (2009; <https://doi.org/10.1373/clinchem.2008.112797>). The abundance of each selected gene will be expressed as gene copy/16S rRNA gene copy. In case of gene concentration values below the LOQ but higher than the limit of the technique (i.e., three gene copy/reaction, Bustin et al., 2009) the sample will be considered as positive but not quantifiable (NQ). Each positive amplicon will be tested by electrophoresis to verify its right size.

## 2.8 ddPCR

ddPCR will be performed in case qPCR analyses will be insufficient in clarifying the presence of AB resistance genes in the analyzed samples. The quantified DNA samples (in a similar concentration) will be tested to quantify selected antibiotic resistance genes (ARGs) and the 16S rRNA gene by digital droplet PCR (ddPCR) following the protocols already described in Di Cesare et al. (2018; <https://doi.org/10.1111/1758-2229.12665>). For each selected gene to test, the program of ddPCR will be set in order to select the best annealing temperature (this could be different than that used in qPCR). At least two positive controls and two NTCs will be tested with the DNA samples in the same reaction. Only reactions with more than 104 droplets were analyzed. The samples will be considered positive if at least three positive droplets will be detected. The concentration of each selected ARG will be expressed in a relative manner as described in the paragraph of the qPCR.

## 2.9 Cytometric analyses

The abundance of pigmented picophytoplankton and non-pigmented heterotrophic planktonic microorganisms will be determined using the Flow Cytometer A50-micro (Apogee Flow System, Hertfordshire, UK), equipped with a 20 mW Solid State Blue Laser (488 nm). Samples will be immediately placed in 2-ml Eppendorf safe-lock tubes, fixed on site with formaldehyde (2% final concentration), and stored in a refrigerated box. The light scattering signals (forward and side light scatter named FSC and SSC, respectively), red fluorescence (> 610 nm), orange fluorescence (590/35 nm), and green fluorescence (530/30 nm) will be acquired and considered for the direct identification and quantification of distinct microbial groups by following common protocols (Gasol and Morán, 2015). Cyanobacteria and pico-eukaryotic algae will be characterized and distinguished according to their pigmentation (i.e., reflecting on different intensities of auto fluorescence signals collected at the orange and red channels) and size (i.e., proportionally related to light scatter signals). Thresholding will be set on the red channel in order to exclude most of the unspecific signals according to 0.2- $\mu$ m filtered control seawater. The gating strategy will be manually adjusted on the density plots of SSC versus Red and of Orange versus Red channels.

The abundance of prokaryotic and nano-eukaryotic cells (e.g., bacteria and nanoflagellates) will be determined by following the staining procedure with SYBR Green I (1:10,000 dilution; Molecular Probes, Invitrogen) for 10 min in the dark at room temperature. Samples will be run at low flow rates to keep the number of detected total events below 1,000 per second. The data handling and visualization will be performed by the Apogee Histogram Software (v89.0).

### 2.10 (Sea)water sampling for the analysis of antibiotics

Amber glass bottles (1 L for mariculture samples and 500 mL for RAS samples) will be pre-rinsed with ultrapure water to remove dust, followed by ethanol and acetone, individually, and then allowed to dry out any remaining solvent overnight. Before sampling (and between sites), bottles will be rinsed again with water to be sampled. Surface water (upper 20 cm) will be collected to avoid contact of water samples with bottle caps by placing a piece of aluminum foil in between. Transport of samples to the lab under cold and darkness conditions (-20°C if possible, 4°C otherwise). Samples will be kept at -20°C until shipment.

### 2.11 Sediment sampling for the analysis of antibiotics

Aluminum trays will be wiped with ethanol and acetone. Grab sediments (upper 10 cm) using a sediment sampler (Van Veen or Ekman sediment sampler) will be transferred to aluminum trays and covered with aluminum foil. Transport of samples to the lab will occur under cold conditions (-20°C if possible, 4°C otherwise) and kept at -20°C until freeze-drying and subsequent transferring of required dry amounts into closed small amber glass bottles for shipment. Freeze-dried sediments will be sieved prior to extractions.

### 2.12 Benthic biota sampling for the analysis of antibiotics

Aluminum trays will be wiped with ethanol and acetone individually. The same procedure will be applied to other tools used for benthic fauna processing, also in between sites. After benthic fauna sampling and classification (e.g., by species), collect pool in aluminum tray covered with aluminum foil. Transport of samples will be performed to the lab under cold conditions (-20°C if possible, 4°C otherwise) and kept at -20°C until freeze-drying. Freeze-dried samples will be transferred into closed small amber glass bottles for shipment. Shipment will be performed in containers with dry ice. Once received in the lab, freeze-dried benthic fauna will be homogenized prior to extractions.

### **2.13 Fish fillet sampling for the analysis of antibiotics**

Aluminum trays will be wiped with ethanol and acetone individually. The same procedure will be applied to other tools used for fish dissection, also in between samples. Fish fillet (without skin; around 50 g wet weight) will be dissected and transferred to an aluminum tray covered with aluminum foil. Transport of samples to the lab will be performed under cold conditions (-20°C if possible, 4°C otherwise).