DEVOTES
www.devotes-project.eu
Development of innovative tools for understanding marine biodiversity and assessing good environmental status

WP 5 | Milestone 22

Optimized protocols for molecular and microarray analyses available

Milestone 22

Dissemination level

Public (PU)

LEAD CONTRACTOR

Consorzio Nazionale Interuniversitario per le Scienze del Mare (CoNISMa)

AUTHORS

Marco Berzano (CoNISMa), Stefano Accoroni (CoNISMa), Laura Carugati (CoNISMa), Annalucia Cantafaro (CoNISMa), Cristina Di Camillo (CoNISMa), Nina Dzhembekova (IO-BAS), Esther Garcés (CSIC), Marco Lo Martire (CoNISMa), Francesca Marcellini (CoNISMa), Snejana Moncheva (IO-BAS), Helen Parry (PML), Chiara Pennesi (CoNISMa), Nadia Pieretti (CoNISMa), Fabrizio Vecchi (CoNISMa), Claire Widdicombe (PML), Stephen Widdicombe (PML), Roberto Danovaro (CoNISMa).

SUBMISSION DATE

21| December | 2015

DEVOTES is co-funded by the European Commission within the 7th Framework Programme 2007 -2013

www.devotes-project.eu
Contract number 308392

Contents

1. Milestone scope 1
2. Microarray protocols 2
3. References 3
4. List of annexes 3

1. Milestone scope

The aim of Milestone 22 "Optimized protocols for molecular and microarray analyses available" is to make available on the DEVOTES website the protocols used to perform microarray analyses described in the Deliverable 5.4 (Report on the optimization of protocols and results of the molecular analyses of biodiversity).

The Deliverable 5.4, published in November 2015, is one of the important objectives of WP5, which aims to apply and evaluate the effectiveness of innovative tools to assess environmental status. Most of the innovative approaches are based on the latest molecular techniques applied to identify different organisms (from microorganisms to large invertebrates, including phytoplankton), belong to different species. In particular, Deliverable 5.4 is related to tasks 5.1.3 ("applying innovative tools to gather information on the biodiversity of potentially-dangerous algae for human health") and 5.2.2 ("validation of the identification of toxic genes of harmful algae") regarding the use of microarray to identify algae potentially toxic for human health. The system of microarray was developed within the MIDTAL European project and consists of a series of genetic probes targeting specific harmful algae of interest (Lewis et al., 2012). The final objective is to gather information on the diversity of potentially-dangerous algae, such as species of dinoflagellates and other key taxa, determining Harmful Algal Blooms (HABs).

Deliverable 5.4 describes the application of microarray system in different European seas (from the English Channel to the Adriatic Sea) in order to gather "real-time" information on the presence of potentially toxic algae. More in detail, the report outlines: (i) the background about the use of microarray; (ii) the protocols used to collect and filter seawater samples and run microarray; (iii) the comparison between the results obtained from light microscope and microarray analyses; (iv) the evaluation of the advantages/disadvantages of this method to identify toxic algae (sensitivity of the probes and analysis of the costs).

2. Microarray Protocols

All the protocols applied to perform microarray analysis described in the Deliverable 5.4 are reported in the Annex I and Annex II and are available in the DEVOTES ftp server at the following link: ftp://www.devotes-project.eu/WP5/Microarray/Protocols/

Annex I contains a short protocol describing activities from seawater sampling to microarray hybridization. Protocols for the following steps are detailed:

i) Field sampling

ii) RNA Extraction

iii) RNA clean-up

  a. Clean-up with an Amicon Ultra-0.5 (30 K centrifugal device, Millipore)

  b. Clean-up with Ammonium Acetate (NH4Ac)

  c.

iv) RNA labeling using the PlatinumBright 647 Infrared (KREAtech)

  a. Cy5 labeling of the samples

  b. Purification of the labeled samples

v) Microarray hybridization

  a. Microarray preparation

  b. Hybridization processing

Moreover, a detailed protocol for seawater sampling and filtering has been reported in Annex II. This is the protocol that each partner involved in Tasks 5.1.3 and 5.2.2 followed to collect and filter seawater. For the scope of the analysis it is important to take note of the following information during sampling activities:

- Sampling location
- GPS coordinates
- Frequency of sampling

- Duration of sampling (months)
- Sampling starting date
- Equipment available
- Storage (-80°C).

3. References

Lewis J., Medlin L.K., Raine R., 2012. MIDTAL (Microarrays for the Detection of Toxic Algae): a protocol for a successful microarray hybridization and analysis. Koeltz, Germany.

4. List of annexes

Annex 1 – **MS22. Annex1_MIDTAL short protocol (.pdf)**

Annex 2 – **MS22. Annex2_Sampling protocol (.docx)**

Short protocol

A. Field sampling

1. Collect up to 10 L of water from your favorite location.
2. Filter one liter of your field sample onto a 1.2 µm, 25 mm nitocellulose or polycarbonate filter using low vacuum pressure (100 mm Hg). Repeat this step 3 times for RNA extraction to produce three replicates. If you want to do DNA extractions, increase your number of filters.

   Note: you can filter more than 1 liter if it is needed but always write down the volume of water you filtered.
3. Put each filter in 1 ml of Tri-Reagent (Sigma) and vortex well.

   Note: if the filter is dark, you must add more Tri-reagent until the colour of your sample is very light brown-pink. Do not forget to vortex well to homogenize your sample: it helps to preserve RNAs in a good quality state.
   Note:You can stop at this step by freezing the sample in Tri-Reagent and keeping it at -80°C but we found a better RNA extraction if you bead-beat the sample(with the Dunaliella control) before freezing it.

   Tri-Reagent is toxic: follow carefully manufacturer's instruction.

B. RNA Extraction

1. Add your extraction control (500 000 *Dunaliella tertiolecta* cells) and vortex well.
2. Add glass 1 to 2 mm of glass beads (mixture of 212-300 and 425-600 µm, Sigma).
3. Bead beat twice for 1 minute at room temperature at maximum speed.
4. Vortex 15 seconds and incubate at room temperature for 5 minutes.
5. Vortex 15 seconds and incubate at 60°C for 10 minutes in a thermomixer at maximum speed or keep vortexing one after the other.
6. Prepare the Phase Lock Gel (PLG) Heavy 2 ml tubes (5Prime) as described by manufacturer.
7. Cool down the sample at room temperature and transfer the supernatant to the PLG tubes.
8. Add 100 µl of 1-Bromo-3-Chloropropane (BCP) and shake vigorously (DO NOT VORTEX)
9. Incubate 5 minutes at room temperature
10. Centrifuge for 15 minutes at 12000 g at 4°C.
    Note: if the phases are not separated enough, do an additional 15 minutes centrifugation.
11. If the aqueous phase is still lightly pink, do an additional BCP extraction by adding I the same PLG tube, 100 µl of BCP. Mix well by shaking vigourously and centrifuge for 15 minutes at 12000 g at 4°C.
12. Transfer the aqueous phase to a new tube
13. Add 0,5 volume of Isopropanol and invert several times.
14. Incubate at -20°C for 1 hour
15. Centrifuge for 15 minutes at 12000 g at 4°C.
16. Quickly remove the supernatant, add 1 ml of 75% Ethanol and vortex 5 seconds
17. Centrifuge for 10 minutes at 12000 g at 4°C.
18. Repeat steps 15 to 17 twice

19. Remove the supernatant and dry the RNA pellet.
20. Resuspend in 20-50 µl of Rnase-free water.
21. Measure RNA quantity and purity. If the 260/230OD ratio is less than 1.8, proceed with the clean up protocol.

A. RNA clean-up

a. Clean-up with an Amicon Ultra-0.5 (30 K centrifugal device, Millipore).

1. Add 350 µl of nuclease free water to the column
2. Centrifuge for 10 minutes at 14 000 g at 4°C
3. Discard flow-through
4. Adjust the sample volume to 400 µl and transfer it to the column
5. Centrifuge for 10 minutes at 14 000 g at 4°C
6. Place the device upside down in a new tube
7. Centrifuge for 2 minutes at 1 000 g at 4°C
8. Adjust the volume to 20-50 µl.
9. Measure RNA quantity, purity and integrity (Nanodrop and Bioanalyzer).
10. Store the sample at -80°C.

b. Clean-up with Ammonium Acetate (NH_4Ac)

1. Adjust the sample volume to 100 µl and add 0.5 volume of 7.5M Ammonium Acetate and 2 volume of 100% Ethanol.
2. Vortex and incubate at -80°C for 1h30.
3. Centrifuge for 20 minutes at maximum speed at 4°C.
4. Remove the supernatant and add 500 ml of 70% Ethanol.
5. Centrifuge for 5 minutes at maximum speed at 4°C.
6. Repeat this washing step once
7. Air dry the pellet and resuspend in 20-50 ml
8. Measure RNA quantity, purity and integrity (Nanodrop® and Bioanalyzer®).
9. Store the sample at -80°C.

B. RNA labeling using the PlatinumBright 647 Infrared (KREAtech)

a. Cy5 labeling of the samples

1. Mix and homogenize 1.5 µg of your target RNA with 2 µl of ULS label and 2 µl of 10X labeling buffer for a final volume of 20 µl.
2. Incubate at 85°C for 30 minutes.
3. Cool down your samples on ice and spin briefly to collect contents at the bottom of the tube.

b. Purification of the labeled samples

1. Prepare the KREApure column by vortexing it.
2. Loose the cap and snap off the bottom closure
3. Place the column in a 2 ml collection tube and centrifuge at 20 800 g for 1 minute at 4°C
4. Discard the flow-through and add 300 µl of nuclease-free water
5. Centrifuge at 20 800 g for 1 minute at 4°C and discard the flow-through

6. Place the column in a new 2 ml tube and add the labeled sample directly onto the column
7. Centrifuge at 20 800 g for 1 minute at 4°C
8. Measure the dye incorporation of your RNA with the Nanodrop and calulate the degree of labeling (DoL; http://www.kreatech.com/products/universal-linkage-systemtm-labeling-kits/microrna/dol-calculation.html).
9. Add 1/10 volume of fragmentation buffer (100 mM $ZnCl_2$ in 100 mM Tris-HCl pH 7) to the labeled sample and incubate at 70°C for 15 minutes.
10. Stop the reaction by adding 1/10 volume of 0.5M EDTA pH 8 and incubating at room temperature for 5 minutes.

C. Microarray hybridization

a. Microarray preparation

1. Mark the array's location with a glass cutter using the provided template.

   Note: All the slides are already blocked and must be kept at 4°C in the dark. They will last for at least 6 months.

b. Hybridization processing

1. Prepare your slide by placing a clean LifterSlips (Thomas Scientific 25 x 25 mm) on each array (use the mark you did with the glass cutter to localize your arrays).
2. Prepare the hybridization mixture by mixing 500 ng of the previously labeled RNA, 4X Hybridization buffer, 1 µl of TBP-control (5ng/µl) and 1 µl of poly dA (1 µM) to a final volume of 30 µl with nuclease free water.
   Note: the 4X Hybridization buffer contains BSA(2mg/ml), Herring sperm DNA (0.4 mg/ml), NaCl (4M), Tris-HCl pH8 (40 mM) and Triton100 (0.02%)
3. Denature the mixture at 95°C for 5 minutes
4. Add 7.5 µl of KREAblock to your mixture and proceed immediately to the hybridization.
5. Add the 30 µl of the hybridization to the arrays and incubate in a wet chamber at 65°C for 1 hour.
6. Before rmoving the coverslips, let the slides cool down at room temperature for 5 to 10 minutes.
   Note: this step avoid non specific background
7. Remove carefully the coverslips and wash the slides in Wash Buffer 1 for 10 minutes at room temperature, Wash Buffer 2 for 10 minutes at room temperature and Wash Buffer 3 for 10 minutes at 50°C.

   Note: Wash Buffer 1: 2X SSC, 10mM EDTA, 0.05% SDS
   Wash Buffer 2: 0.5X SSC, 10mM EDTA
   Wash Buffer 3: 0.2X SSC, 10mM EDTA

8. Transfer quickly the slides from the last bath to a slide rack and centrifuge at 900 rpm for 3 minutes at room temperature
   Note: at this stage, no traces should be seen
9. Scan immediately with a microarray scanner.

Annex 2: Schematic overview of DEVOTES sampling protocol:

- Sub-surface water samples (from two depths in the range of 5-10 m and at the thermocline, surface water samples are not recommended) can be taken using a simple water sampling bottle (Niskin or similar)*
- Collect up to 10 L of the mixed sample, pre-filter it with a 200/250 µm mesh net and bring it to the laboratory for filtration
- One or Two aliquots of 250ml of seawater have to be stored in brown bottles and fixed with 1ml of Lugol ʻs solution** or Formaldehyde (0.8%) for subsequent microscopic identification and enumeration
- Samples should be preserved at *in situ* temperature immediately after collection and during short-term storage to the laboratory.

* if a sampling boat is not available, samples can be taken from the shore/pier

° if Lugol is not easily available, formaldehyde 0.8% could be used.

**[lugol solution: Dissolve 50g potassium iodide (KI) and 25g iodine (I2) in 100 ml boiling water (= solution A). Dissolve 25 g sodium acetate (CH3COONa) in 250 ml water (= solution B). When solution A cools, mix solutions A and B and store in a cool dark place].

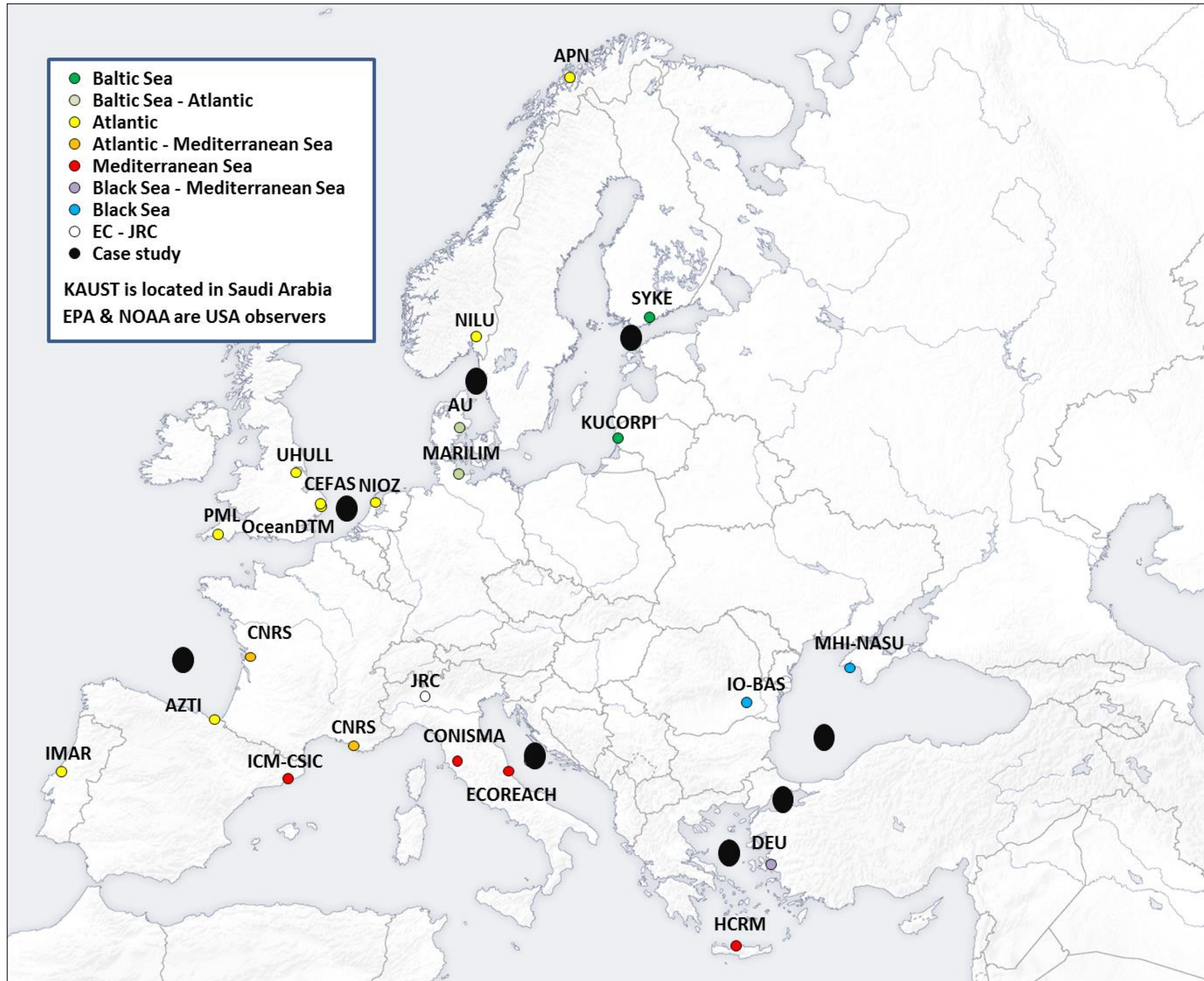
Filtering the collected seawater:

- Filter between 500ml and 1L of seawater on 3µm Cellulose Nitrate filters (**25mm ⃠**) [less than 400ml will require an additional set of 3 filters for RNA extraction]
- Record the volume of filtered seawater **per each filter (n=6 filters)**
- We require **3 filters** for RNA analysis and **3 filter** for DNA analysis
- Manifold filtration unit with filter funnels and a vacuum pump is recommended
- One or Two bottles (250ml) of pre-filtered seawater in fixative for enumeration and identification (specify the methodology as well as the type of chamber employed)
- 1mL of Tri-reagent (Ambion®) solution ONLY FOR **RNA** filters
- 2ml cryovials or screw-cap microtubes (with o-ring)
- Filtered samples should be directly frozen in liquid nitrogen or after being covered with 1ml of Tri Reagent and then stored at -80°C
- Shipping in **dry ice** or using a **dry shipper**

Note:

If you are not able to find the same filters we required to use for sampling, please let us know and we will provide you a sufficient number of filters by post. The same applies to Tri Reagent solution.

Participant no./short name	Sampling location	GPS coordinates	Frequency of sampling	Duration of sampling (months)	Sampling starting date	Equipment available	Storage (-80°C)
CONISMA	Adriatic Sea	XX XX	6 per year	12	March	Niskin	yes



Notes and comments: