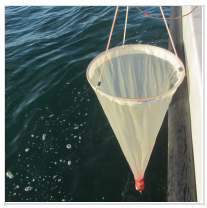

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Oct 13, 2021

Environmental DNA sampling protocols for the surveillance of marine non-indigenous species V.2

1

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Luca Mirimin

This research Initiative (SERV-19-MEFS-004) is part of a wider series of ongoing technical studies to support marine spatial planning and related Marine Strategy Framework Directive decision making. The Blue Growth & Marine Spatial Planning Scheme is established under Union Priority 6 (Integrated Maritime Policy) of Ireland's European Maritime and Fisheries Fund (EMFF) Operational Program. It is co-funded by the Irish Government and the EU.

This document describes a series of protocols for the collection of environmental samples intended for the monitoring and surveillance of marine invasive species by means of eDNA metabarcoding analysis, as described in the associated publication (Fernandez et al. 2021: https://doi.org/10.1016/j.marpolbul.2021.112893).

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Luca Mirimin

protocol

Sara Fernandez, Dulaney L. Miller, Luke E. Holman, Arjan Gittenberger, Alba Ardura, Marc Rius, Luca Mirimin, Environmental DNA sampling protocols for the surveillance of marine non-indigenous species in Irish coastal waters, Marine Pollution Bulletin, Volume 172, 2021, 112893, ISSN 0025-326X, https://doi.org/10.1016/j.marpolbul.2021.112893.

eDNA, sampling protocols, metabarcoding

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In steps of

eDNA/eRNA extraction protocols from marine samples

Protocols for eDNA/eRNA extraction from marine samples

General materials

- Risk assessment protocol and associated materials/equipment (e.g. life-jackets, waterproof gear, etc.)
- Instrument to record GPS coordinates (e.g. a mobile phone)
- Metadata recorder (e.g. clipboard with waterproof paper and pencil, or mobile phone/tablet, etc.)
- 20% Sodium hypochlorite (commercial bleach)
- 70% and absolute ethanol
- Large black bin bags
- Spray bottle (to be used with bleach)
- Clean paper roll
- Disposable gloves
- Permanent markers
- Sellotape (to cover writing on tubes)
- Large cooler box or dark buckets with lids (for short term sample storage) (OPTIONAL)
- Access to a -20°C freezer (for medium-long term sample storage) (OPTIONAL)
- Multi-parameter reader with probes (e.g. temperature, depth, salinity, DO, pH, etc.) (OPTIONAL)

Materials for collection of water samples (low volume water)

- DNA-free water (for field negative)
- Whirl-Pak bags (e.g. https://whirl-pak.com/whirl-pak-stand-up-thio-bags-2)
- Whirl-Pak pole and adapters/clamps (OPTIONAL) (e.g. NASCO Whirl-Pak® 12 ft. Adjustable Sampling Pole for Whirl-Pak B00679, B00992 and B01062 Bags - B01367WA - Ferguson)
- Niskin bottle (OPTIONAL) (e.g. Niskin / PWS 3.5 l | HYDRO-BIOS (hydrobios.de))
- DNA-free 15ml falcon tubes
- Silica beads (e.g. Silica gel (2.5-6.0mm) self indicating, dessicant agent, Fisher Chemical™ 5kg, Plastic keg Products | Fisher Scientific)

Filtration

Citation: Environmental DNA sampling protocols for the surveillance of marine non-indigenous species https://dx.doi.org/10.17504/protocols.io.byz2px8e

- Portable vacuum pump and battery
- Tubing for vacuum filtering (e.g. Clear Silicone Tubing Hose Pipe Pond Aquarium Fish Tank Air Pump AFS Tube FDA | eBay)
- Disposable filters and funnels
- DNA-free reusable (or disposable) forceps
- DNA-free 5-50ml tubes or small zip-lock bags for filter storage
- Multiple filters funnel manifold (OPTIONAL) (e.g. Modular Filter Funnel Manifold, Pall Laboratory | VWR)
- Ice blocks for storage (for onsite filtration) (OPTIONAL)
- Fixative (e.g. silica beads, RNAlater or Longmire solution) (OPTIONAL)

Materials for collection of water samples (high volume water)

- Mark II inDepth eDNA sampler and associated battery charger/reader (Applied Genomics, Brixham, UK; Biodiversity and eDNA Survey, Analysis and Monitoring | Applied Genomics)
- DNA-free moorings consisting of anchor, sinkable ropes and floats
- 1 µm polyethersulfone large volume filter capsules (Effective Filtration Area 1300 cm^2)
- Tubing and one-way valve (for filter capsule's inflow)
- Fixative solution (Applied Genomics, Brixham, UK)
- DNA-free funnel (to pour fixative into filter capsule)
- Parafilm
- DNA-free large syringe with tubing or hand pump (OPTIONAL)
- Synthetic DNA Internal Positive Control (OPTIONAL)
- Sterile 1ml syringe (to inject fixative) (OPTIONAL)

Materials for collection of water samples (high volume tow net)

- 100% DNA-free ethanol (for field negative)
- Squeeze bottle (to rinse/flush net with ethanol)
- DNA-free tow nets with handle bar and collection bucket assembly (e.g. Aquatic Research Instruments - Equipment and services for Aquatic Research)
- DNA-free 50ml falcon tubes
- Flow meter (OPTIONAL) (e.g. Mechanical Flow Meter with back run stop | HYDRO-BIOS (hydrobios.de))
- 50 m measuring cord (OPTIONAL)

Materials for collection of sediment samples

- Box corer or Ekman Grab with weights (e.g. http://www.duncanandassociates.co.uk/equip.htm#box)
- Rope
- 100% DNA-free ethanol (for field negative)
- Squeeze bottle (to rinse/flush net with ethanol)
- DNA-free 50ml falcon tubes
- Silica beads (e.g. Silica gel (2.5-6.0mm) self indicating, dessicant agent, Fisher Chemical™ 5kg, Plastic keg Products | Fisher Scientific)

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MAKE SURE TO ESTABLISH A ROBUST AND THOUGHT THROUGH RISK ASSESSMENT TO MINIMIZE RISK OF HARM TO USERS AND OTHER PARTIES

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MAKE SURE THAT BIOSECURITY MEASURES ARE PUT IN PLACE TO AVOID THE UNINTENTIONAL SPREAD OF HARMFUL OR UNWANTED ORGANISMS

HEALTH AND SAFETY

1

MAKE SURE TO ESTABLISH A ROBUST AND THOUGHT THROUGH RISK ASSESSMENT TO MINIMIZE RISK OF HARM TO USERS AND OTHER PARTIES

Protocol for collection of water samples (low volume water)

2 *SAMPLING*

This can be done from a boat, floating or fixed structure, depending on the goal of the survey.
For each sample/location, make sure to record all associated metadata; e.g. date, time, location, Rep #, other code, operator name, environmental parameters, etc.

2.1 Put on clean, single-use gloves.

Change gloves if a glove has contacted anything except the sampled water body or decontaminated equipment. For example, if your glove touches your clothes, skin, etc., change gloves. For ease of changing gloves regularly, it is advised to use double-gloves (e.g. only outer glove is replaced).

2.2 For each site, pre-label the appropriate number of Whirl-Pak bags as follows:

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https://dx.doi.org/10.17504/protocols.io.byz2px8e

1. "Site name/code", "Date", "LV Water field blank"
2. "Site name/code", "Date", "Rep 1"
3. "Site name/code", "Date", "Rep 2"
4. "Site name/code", "Date", "Rep 3"
...
n. "Site name/code", "Date", "Rep n"

2.3 Take the pre-labelled field blank Whirl-Pak bag and fill with DNA-free water at the site location.

This will be the field negative control sample. Keep sample blank in a cool, dark place (e.g. black bin bag or cooler box) until you are ready for filtration either in the filed or in the lab.

2.4 Take the next pre-labelled Whirl-Pak bag (e.g. "Rep 1") and fill with site water either from the surface (using a DNA-free pole and bag adapter) or at depth by using a DNA-free niskin bottle.

If using a Niskin bottle, it is recommended to rinse 3 times with site water prior to sample collection.

It is also recommended to take at least one additional sample than intended, to account for potential loss of samples during transport/filtration.

Make sure to seal bags properly and keep them up right in a cool dark place during transport and prior to filtration.

2.5 Repeat Step 1.4 as many times as the number of samples required from a given site/location.

Record all metadata (e.g. "Site name/code", "GPS", "Date", "Rep #", "time of deployment", etc.).

2.6 (OPTIONAL) If available, use a water parameter meter to record temperature, salinity, depth, pH, DO, etc.

Record all metadata.

3 *GEAR DECONTAMINATION*

This step is necessary only for re-usable gear.

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3.1 While it is not necessary to decontaminate gear between replicate samples within a given site/location, it is necessary to decontaminate any reusable equipment or accessory prior to sampling the next location.

Wipe the Whirl-Pak pole and spray clamps and adapters with 20% bleach and leave to decontaminate for at least 15 minutes.

To reduce sampling time and risk of cross site contamination, it is recommended to have several pre-prepared sets of clamps and adapters (e.g. ideally as many as the intended number of sites to be visited).

4 *WATER FILTRATION*

This step can be done in the field or in a dedicated "DNA-free" laboratory.
Note that the peristaltic pump can be replaced with alternative options (e.g. manual pump or drill connected to a peristaltic pump head).

4.1 Put on clean, single-use gloves and wipe clean the surface work area with 20% bleach. For ease of changing gloves regularly, it is advised to use double-gloves (e.g. only outer glove is replaced).
If filtering in the field, use a disposable or reusable plastic sheet (e.g. tarpaulin) as surface.

Set up the filter pump, power source (mains, battery, car) and (if available) the multi-filer manifold, ensuring that all connections are tight and that the flow of water/air will follow the intended direction (e.g. away from the filters).

4.2 Pre-label 15 mL falcon tubes (pre-filled with 4mL silica beads) following the same labeling as that suggested in Step 1.2. It is recommended to secure the label by covering it with a strip of sellotape to avoid risk of mislabeling.

4.3 Place DNA-free adapter-funnel-filter assemblies in the tubing (or manifold), ensuring that the funnel is kept vertical and is secure (to avoid any spillage).

Filter each sample gradually, ensuring that any suspended particle is also poured into the funnel by swirling the liquid in the Whirl-Pak bag. Using DNA-free forceps, remove the filter from the funnel (top membrane, not the lower thick support pad), fold onto itself to create a semi-circle shape, and place in its respective pre-labelled falcon tube. Store tubes in a cool dark place (for short-term storage) and place in a -20°C as soon as possible (for medium-long term storage).

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Record metadata (e.g. time of filtration of each sample).

Protocol for collection of water samples (high volume water)

5 *DEPLOYMENT AND RETRIEVAL*

Note that the location of deployment should be chosen to ensure that at least 2 meters of water are present at low tide and that it will not interfere with local boat traffic.

5.1 Put on clean, single-use gloves.

Change gloves if a glove has contacted anything except the sampled water body or decontaminated equipment. For example, if your glove touches your clothes, skin, etc., change gloves. For ease of changing gloves regularly, it is advised to use double-gloves (e.g. only outer glove is replaced).

5.2

Ensure that all equipment has been decontaminated with 20% bleach prior to deployment.

Check that the sampler's battery has sufficient charge.

Deploy the sampler by inserting and securing the starter plug prior to releasing in the water. Note that the sampler inflow valve should be facing upward.

Record all metadata (e.g. "Site name/code", "GPS", "Date", "Rep #", "time of deployment", etc.)

5.3 Retrieve the sampler after the filtration cycle has completed (e.g. 25 hours).

Remove the filter capsule and remove any excess water through the outflow (not inflow). This step can be done either using a pump or large syringe or by gravity, but making sure to use DNA-free equipment/materials to avoid potential contamination of the sample.

Fill the capsule with fixative, inject 1 mL of Internal Positive Control (OPTIONAL), and seal both ends using caps and/or Parafilm. Label each sample and store the sample in a cool dark place for short-medium term until extraction.

If a field negative is required, fill a filter capsule with fixative and IPC while on

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5.4 site and store/process as per other samples.

Protocol for collection of water samples (high volume tow net)

6 *SAMPLE COLLECTION*

The choice of the sampling location is determined by the goal of the survey but also by the intrinsic limitations of the equipment used. The following protocol has been designed to collect "plankton-like" tow samples either by hand (using an extension pole attached to the side of the net opening) or by boat (attaching the net to the boat with a rope).

6.1 Put on clean, single-use gloves.

Change gloves if a glove has contacted anything except the sampled water body or decontaminated equipment. For example, if your glove touches your clothes, skin, etc., change gloves. For ease of changing gloves regularly, it is advised to use double-gloves (e.g. only outer glove is replaced).

6.2 Prior to deployment, each net and other reusable equipment must be decontaminated in order to remove any DNA trace form previous use. While most materials and small equipment can be easily decontaminated by soaking in 20% bleach for at least 20 minutes (with occasional mixing/turning), it is recommended that nets must be fully submerged in bleach in a large deep tray and rinsed with DNA-free water at least 3 times while rubbing and "massaging" the net and seams by hand to remove any excess bleach.

6.3 For each site, pre-label the appropriate number of 50 mL falcon tubes as follows:

1. "Site name/code", "Date", "Net field blank"
2. "Site name/code", "Date", "Rep 1"
3. "Site name/code", "Date", "Rep 2"
4. "Site name/code", "Date", "Rep 3"

...

n. "Site name/code", "Date", "Rep n"

It is recommended to secure the label by covering it with a strip of sellotape to avoid risk of mislabeling.

6.4 Using the 100% ethanol squeeze bottle, rinse the pre-decontaminated net and collect 40-50 mL of liquid in the falcon tube labeled as "Net field blank".

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6.5 For each subsequent sample, tow the net for the intended distance/time (e.g. 50 meters transects) and making sure that the net is fully submerged. Prior to retrieval, make sure to concentrate most of the suspended material trapped in the net into the dolphin bucket by performing several vertical “dips”.

Remove as much excess water as possible by holding the net at an angle and letting the water sip through the mesh of the dolphin bucket. If this is taking a long time, close off a portion of the net just above the dolphin bucket, and use the additional surface area to drain water. Draining the water is essential to have proper preservation of the eDNA.

Using the ethanol squeeze bottle, rinse down the bottom of the net into the dolphin bucket and detach the dolphin bucket. Then, rinse the dolphin bucket screens and transfer material to the 50 mL tube. Keep the net high in the air while rinsing down to keep ethanol in the dolphin bucket. If necessary, split the sample into an additional falcon tube. Aim for at least 80% ethanol in each sample for adequate preservation.

6.6 Store samples out of direct sunlight, preferably on ice and in a rack. Keep the samples upright as much as possible to prevent leakage and contamination.

Protocol for collection of sediment samples

7 *SAMPLE COLLECTION*

Note that the choice of sampling location is dependent on availability of suitable substrate (e.g. soft sand) and that the sampling success rate will also depend on the quality and appropriateness of the gear used (e.g. heavy box corer).
Warning: use Silica beads in a ventilated outdoor area or under a hood. Do not touch or inhale Silica beads.

7.1 Put on clean, single-use gloves.

Change gloves if a glove has contacted anything except the sampled water body or decontaminated equipment. For example, if your glove touches your clothes, skin, etc., change gloves. For ease of changing gloves regularly, it is advised to use double-gloves (e.g. only outer glove is replaced).

7.2 Ensure that all equipment has been decontaminated with 20% bleach prior to deployment.

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For each site, pre-label the appropriate number of 50 mL falcon tubes as follows:

1. "Site name/code", "Date", "Sediment field blank"
2. "Site name/code", "Date", "Rep 1"
3. "Site name/code", "Date", "Rep 2"
4. "Site name/code", "Date", "Rep 3"
...
n. "Site name/code", "Date", "Rep n"

It is recommended to secure the label by covering it with a strip of sellotape to avoid risk of mislabeling.

7.3 Using the 100% ethanol squeeze bottle, rinse the inside of the decontaminated box corer and capture 40-50 mL of liquid in a falcon tube labeled as "Sediment field blank".

Store the sample blank out of direct sunlight, preferably on ice and in a rack. Keep the sample upright as much as possible to prevent leakage and contamination. Note that only the blank sample will be stored using ethanol, and will have to be extracted using a separate protocol from the actual sediment samples.

7.4 Deploy and retrieve the box corer. Let any seawater on top of the sediment drain off and then use a falcon tube to scoop off approximately 10 mL of sediment from the top layer of sediment (approximately 1-2 cm).

7.5 Rinse with site water and redeploy for the next replicate sample.

Repeat the above steps until the intended number of replicate samples has been collected.

7.6 Fill sediment tubes with 30 mL of Silica beads on top of the sediment or more if the sediment sample exceeds 10 mL (3:1).

Store tubes in a cool dark place (for short-term storage) and place in a -20°C as soon as possible (for medium-long term storage).

Record metadata (e.g. "Site name/code", "GPS", "Date", "Rep #", "time of deployment", etc.).

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