Guidelines for the monitoring of target non-indigenous species using molecular methods

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1. Background

1.1 Introduction

Marine biological invasions are increasingly changing coastal biota. They can alter ecosystem functioning and sometimes seriously affect an economy and human health, and so remain high on the environmental management agenda. The HELCOM Baltic Sea Action Plan recognizes the issue in its Management Objectives for Maritime Activities: "No introductions of alien species from ships". Monitoring of non-indigenous species (NIS) is required by several international agreements and guidelines, such as the Biodiversity Strategy, Marine Strategy Framework Directive (MSFD) and EU Invasive Species Regulation (EU IAS). NIS data is needed to assess the effectiveness of legal and administrative measures aimed at prevention of unwanted human-mediated introductions, update HELCOM core indicator and to report for EU MSFD, EU IAS regulation, for those HELCOM countries being EU members, and to fulfil the data needs for exemptions applied from the Ballast Water Management Convention (BWMC). NIS monitoring is to address all biotic components as NIS may belong to any trophic level and be found in various man-made as well as natural habitats.

1.2 Purpose and aims

These guidelines describe the use of molecular methods for detection of target non-indigenous invasive species. The "target species" are those that "meet specific criteria indicating that they may impair or damage the environment, human health, property or resources and are defined for a specific port, State or biogeographic region (IMO, 2007)". The criteria and procedure for selecting target species are based on the analysis of their relationship with the introduction vector (e.g., ballast water), the basic ecology of the species and their impact on local species, the environment, human health and resource users (Olenin et al. 2016; Gollasch et al., 2020). The Baltic Sea countries have agreed on the list of target species for the Baltic Sea within HELCOM. The list is being continuously revised and updated by a dedicated Correspondence Group.

2. Monitoring methods

2.1 Monitoring features

1. The protocol is used to reliably and consistently collect and concentrate eDNA from water samples.
2. Water (plankton) sampling leads to a higher probability detecting target species based on eDNA-methods, comparing to e.g. sampling from sediments or settlement plates (von Ammon et al. 2019; Wood et al. 2020), and in combination with the traditional surveillance methods increases efficacy of target species detection (von Ammon et al. 2018). This method is particularly useful for early detection of incursions and screen for species distribution range across large special scales.
3. Collecting and analyzing water samples containing DNA of the organisms living in the area is quicker, easier and cheaper than traditional field DNA collection methods that require scientists to physically contact the animal or plant of interest and take a direct tissue sample from it.
4. Although the strength of eDNA signal can be related to species abundance, a positive eDNA signal does not allow precise evaluation of population abundance or distribution range. However, combining eDNA

results with appropriate statistical modelling tools can allow inference of the likelihood of the target species 'occupancy' of a particular location, such as a marina, embayment or larger surrounding bay or harbour (MacKenzie et. al 2002; Nichols et al. 2008). For absolute confirmation of species detection, it is always recommended that positive signals from eDNA samples are followed up by visual searches.

5. The method does not provide any information regarding factors such as the life stage, reproduction and fitness of a species.
6. Adjustments to this protocol may be necessary, depending on target taxa or environmental conditions of the system being sampled.

2.2 Time and area

Sampling should be conducted during vegetation or reproduction season of a target organism. The spatial design of the sampling varies depending on the purpose of the monitoring, either the detection of rare species or the assessment of species richness. For rare species, sampling in a preferred habitat increases the likelihood of detection. Therefore, knowledge of the ecology and behavior of the target species is critical to the successful application of the eDNA method.

Sampling locations should be selected according to the habitat preference of the target species, taking into hydrodynamic peculiarities of the sampling area, operating introduction pathways nearby and history of the target species introduction (if available). Since eDNA is not distributed homogeneously in water, sampling at different points within the study area increases the likelihood of collecting eDNA released from the target species. A minimum three sample replicates should be collected at each selected sampling site. A chronological order of the sampling sites should be maintained for comparable results.

2.3 Monitoring procedure

2.3.1 Monitoring strategy

Monitoring is conducted by a field crew (min 2 people) from a boat or onshore structures. The field team should be experienced in operating plankton sampling gear and familiar with eDNA sample protocol provided below.

Contamination prevention must be warranted to avoid contamination of a sample and cross-pollution of samples taken in different places. For field sampling, non-DNA materials should be used at each site and for each sample (e.g. gloves, sample kits). Materials that are used in several locations must be thoroughly cleaned between places. Inclusion of negative and positive controls is crucial for reliable outcomes.

Sampling sites in port areas may require a permission from local authorities.

2.3.2 Sampling methods and equipment

Material needed for sampling

- Two plankton nets (20 µm mesh size recommended) complete with ballasted collector, lead ballast and pulling line;
- Labelled sample collection bottles, sterile or bleached before use;
- Squeeze/wash bottle;
- Two large (20 L) buckets for disinfecting/rinsing net, and other material;
- Bleach (~3 L of bleach per sampling campaign);
- Sterile (laboratory) gloves;

- Rubbish bag (for disposing gloves and other litter);
- Chilly bin(s) and ice/ pre-frozen ice packs

Sample collection

Sample are collected by concentrating seston from the water columns via horizontal or vertical plankton net tows. For vertical tows from a boat, the plankton net is towed for at least 1 min at maximum of 2 knots speed (making sure the tow stays at least 1 m depth). Towing time might be adjusting depending on conditions/water turbidity, with obtained signal strength (eDNA copy numbers per sample volume) recalculated accordingly. Vertical tows can be performed from pontoons and berths, at a minimum 2 m depth.

After recovering the net, it should be rinsed with sea water from the sampling site (using the wash bottle), so that particles attached to the net fall into the collector (i.e. cod-end). The collected material is then transferred to sample collection bottles and kept on ice until delivered to a laboratory (as soon as possible).

Between replicates at the same site, rinse the gear (net, cod-end, wash bottle) with the ambient water. Between sampling locations, the gear should be soaked in 2% bleach solution for at least 20 min, and then thoroughly rinsed with the water from the sampling location. If sampling is intended at closely located sites (<20 min transfer time), using second set of gear (bleached and rinsed as described above, using another bucket) is recommended, to ensure that all materials are appropriately cleaned between sites.

'Sampling controls' – at one site (it doesn't matter which) immerse the control bottles in the sea (leaving lids closed) and place back in chilly bin with other samples.

It is recommended to wear gloves when operating the sampling gear and handling the samples and change them between sampling locations.

The following information is to be recorded in the log sheets for each sample collected:

- Date
- Time
- Sample label (site/replicate)
- GPS location
- Depth (for vertical tows)
- Towing time, boat speed and direction (for horizontal tows)

Additionally, we recommend taking notes on Secchi depth, temperature, salinity and pH within sampling area.

2.3.3 Sample handling and analysis

Material needed for lab filtration

- Tweezers (2 or 3 sets recommended)
- Sterile (laboratory) gloves
- Glass microfiber Whatman filters, grade GF/C (1.2 µm pore size, 47 mm dia.)
- Vacuum pump (we recommend using six-way filtering system (Fig. 1))
- Sterile tubes 1.7-2 mL. We recommend splitting each sample into 2 aliquots for back-ups, so prepare double number of tubes: (samples + field blanks)*2. Note: more tubes might be needed if samples are turbid and several filters are used for one sample.
- Scissors

- 2% bleach solution

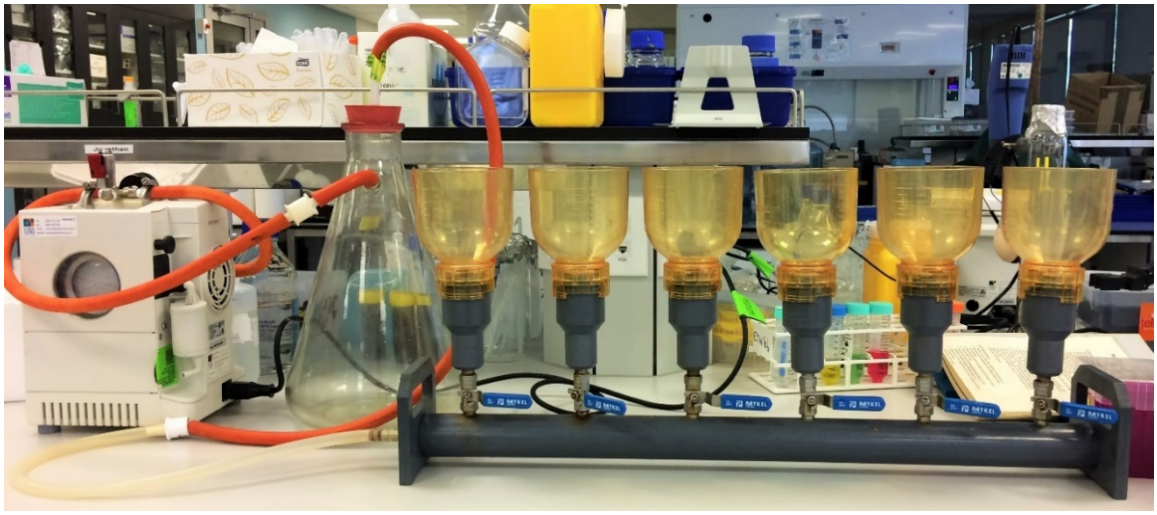

Figure 1: Six-way filtering system for lab-based filtering of water samples (photo: A. Zaiko).

Filtration process

The filtration to be performed as soon as possible (not later than within 24 hours) after sample collection. Make sure that samples are kept chilled until filtration. The working surfaces (i.e. laboratory bench) and filtration gear (cups, and filter holders of the filtration unit, tweezers and scissors with 2% bleach solution) must be cleaned with 2% bleach solution, rinsed (wiped) well with tap water and dried with a clean paper tissue before the process and also between processing samples from different sampling sites.

After sample material is deposited on the filter membrane, it is split into 2 aliquots by cutting it in half with clean scissors. With two tweezers fold the half-filter to reach a quarter of the diameter size (the filtered material should stay inside the folded filter) and place into one of the pre-labelled ~2 mL tube. Repeat for the other half filter (i.e. there should be two tubes per filter). Store the samples at -20˚C immediately until further processing. Repeat for all samples (including field blank controls).

Wear gloves and keep them on throughout the following procedure. Consider changing if samples spill on them or other contamination is suspected.

DNA extraction

The ability to extract good quality DNA from eDNA samples is an important factor in application of specific molecular markers. Since different matrices show great variation in their origin, it is impossible to define a single DNA extraction technique that is successful for all types of eDNA.

For DNR isolation from water samples we recommend PowerWater DNA Isolation Kit because it can isolate genomic DNA from a variety of filtered water samples. Utilizing the patented Inhibitor Removal Technology (IRT), even water containing heavy amounts of contaminants can be processed to provide DNA of high quality and yield. The kit can isolate DNA equally as well from any commonly used filter membrane type.

Species-specific Polymerase Chain Reaction (PCR)

A regionally validated species-specific PCR assay should be used for target detection. End-point, real-time or droplet digital PCR approach can be employed for qualitative or semi-quantitative NIS surveillance (Zaiko et al. 2018). Here we present an example PCR protocol for targeted detection of the invasive mollusk *Rangia cuneata.*

For the detection of *R. cuneata* as a target species, we recommend using species specific molecular marker developed by Ardura et al (2015). This marker is based on presence/absence of PCR amplification product of

target species. The amplification reaction of fragment employed the primers RC-16Sar (5'-AAATTTCTTCTAATGATGTGAGG -3') and 16Sbr (5'- CCGGTCTGAACTCAGATCACGT - 3') and was performed in a total volume of 20 ml, with Promega (Madison, WI), Buffer 1x, 2.5 mM MgCl2, 0.25 mM dNTPs, 20 pmol of each primer and approximately 20 ng of template DNA and 1 U of DNA Taq polymerase (Promega). The following PCR conditions were used: initial denaturing at 95 °C for 5 min, 35 cycles of denaturing at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 7 min.

3. Data reporting and storage

All collected data should be gathered into national monitoring databases. In addition, new non-indigenous species records should be reported to AquaNIS. Before entering the data in a database for introduced and cryptogenic species, it is recommended that presence and distribution of a NIS is confirmed by visual assessment.

4. Contacts and references

4.1 Contact information

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4.2 References

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