Guidelines for the monitoring of non-indigenous species in ballast water of ships, which are accessible by molecular methods

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1. Background

1.1 Introduction

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). In addition, the HELCOM Baltic Sea Action Plan recognizes the issue in its Management Objectives for Maritime Activities: "No introductions of alien species from ships". Non-indigenous species (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. 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).

1.2 Purpose and aims

Molecular techniques are advancing rapidly and are increasingly promoted for NIS monitoring in aquatic systems (Rius et al. 2015; Zaiko et al. 2015a; Viard et al. 2016). These methods are particularly useful for early detection of unwanted organisms, identification of putative NIS, surveillance of high-priority pest species, determination of the source and pathways of invasion, as well as the genetic structure of the founding populations. The advantage of molecular approaches is in their ability to detect and identify NIS at early (dispersible) life-stages and initial stages of invasions, when populations are sparsely distributed and occurring at low densities. Although results of many recent studies have indicated that molecular (eDNA/eRNA-based) approaches show potential as indicative ballast water screening tools (Zaiko et al. 2015b; Darling and Frederick 2017) further research and development is recommended before these methods can be considered for routine application, to improve the representativeness of sampling, better understanding of the longevity of eDNA and eRNA in seawater and ballast water, reference barcode library for invasive marine species of high regional and international priority and quantitativeness of eDNA/eRNA-based assessments to more explicitly address the BWMC requirements.

Yet, molecular screening approaches can be readily applied for, e.g. assessing risks of regional vessel-mediated spread of high-risk NIS, pre-boarder detection of unwanted organisms and justification for granting exemptions from the BWMC at the regional scale. These guidelines describe the use of molecular methods for onboard ballast water monitoring within the aforementioned context and should not be considered as a tool for the BWMC enforcement.

2. Monitoring methods

2.1 Monitoring features

1. Used to detect NIS and other risky organisms (e.g. pathogens) transferred via ships ballast water
2. Method allows to target not only DNA but also RNA that is a better proxy of living (biologically active) organisms
3. Method enables detection of target NIS and pathogens as well as generalized community screening
4. Laboratory analyses require specific skills, laboratory facilities and equipment; sampling can be performed by personnel familiarized with the sampling protocol

2.2 Time and area

Ballast water sampling is recommended before ballast water discharge at port.

2.3 Monitoring procedure

2.3.1 Monitoring strategy

Due to substantial heterogeneity of ballast water and uneven distribution of associated biota, well-designed sampling with appropriate replication is imperative for delivering robust testing results. A minimum three sample replicates should be collected. The sequential filtration of ballast water is not critical for molecular-based monitoring, as it allows to differentiate target groups of organisms by applying different primer sets. Effective sterilization of sampling gear and control for potential contamination when performing on-board testing is essential.

2.3.2 Sampling methods and equipment

High variability in structural characteristics of vessels and their ballast tanks, as well as different access to ballast water sampling points constrain development of universal ballast water sampling design. At this time, we recommend following the ICES *Standard Operating Procedures for Collection of Treated Ballast Water Samples* (ICES 2017). Ballast water samples collected on-board should be kept on ice until delivered to a laboratory (as soon as possible).

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. Pore size should be chosen based on the target fraction of organisms, e.g. 0.2-0.45 um membranes are recommended for bacteria and microbial pathogens, larger pore size (>10 um) membranes can be used for algae and metazoans.
- 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.
- Scissors.
- 2% bleach solution.

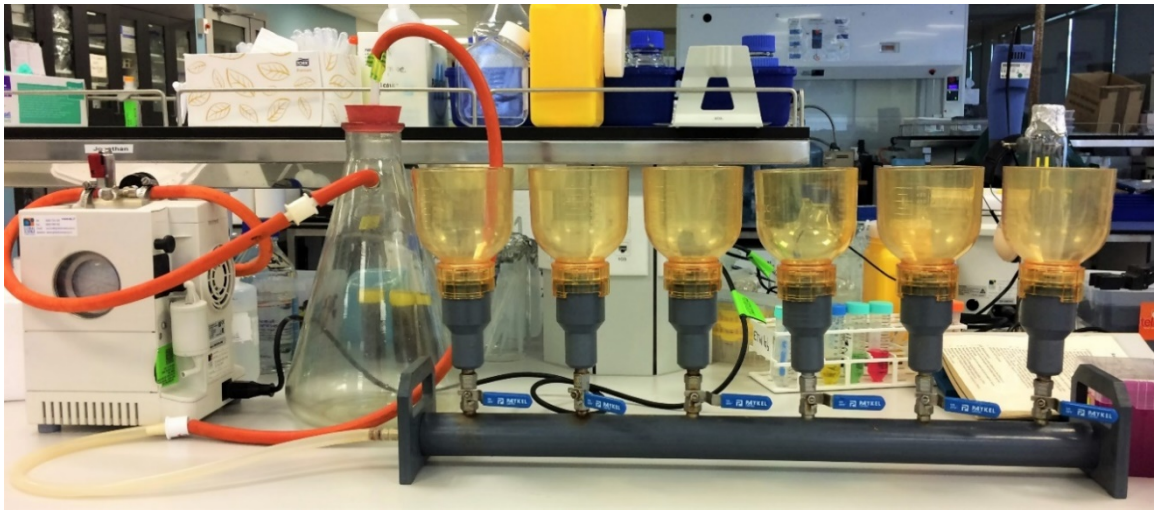

Figure 1: Six-way filtering system for lab-based filtering of water samples (photo: A. Zaiko).

Filtration process

For eRNA analyses, filtration should be performed immediately onboard, and the filter is snap-frozen or preserved with an appropriate buffer solution. For eDNA analyses, the filtration to be performed not later than within 24 hours after sample collection. The working surfaces 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 different samples.

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 (-80˚C for RNA) immediately until further processing.

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

DNA extraction and further processing

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. If RNA analysis is considered, we recommend co-extract DNA and RNA using ZRDuet™ DNA/RNA MiniPrep Kit Plus (Zymo Research, CA, United States), following the sample preparation and reverse-transcription procedure as described in von Ammon et al 2019.

The DNA extracts (and cDNA) then can be analyzed in different ways, depending on the particular monitoring objective and regional risk priorities. These might include: single species (i.e. targeted) assays such as qPCR and digital droplet PCR (ddPCR); enrichment approaches for screening of particular taxonomic groups (i.e. metabarcoding); and whole community analyses using shotgun sequencing. More detailed description of these methods and their application for addressing different marine biosecurity questions is provided in Zaiko et al. 2018. Depending on the chosen approach, an established and scientifically validated analytical protocol should be followed (see e.g. Wood et al. 2019 for qPCR or ddPCR assays; or von Ammon et al. 2018 for metabarcoding).

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 identity of a putative NIS (when derived from metabarcoding analyses) is confirmed by a validated targeted assay.

4. Contacts and references

4.1 Contact information

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4.2 References

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