Guidelines for the monitoring of non-indigenous species in biofouling, which are accessible by 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

Molecular techniques are advancing rapidly and are increasingly promoted for NIS monitoring in aquatic systems (Rius et al. 2015; 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.

These guidelines describe application of molecular (eDNA-based) surveillance techniques for NIS detection and monitoring from early biofouling, i.e. biofilms - "thin layer of bacteria, microalgae, detritus and other particulates that is required for settlement of the larvae of many species of marine invertebrates" (Floerl et al. 2005).

2. Monitoring methods

2.1 Monitoring features

The biofilm method is recommended to quickly detect NIS at the border from biofouling on ship hulls or other floating structure (e.g. for identifying bioinvasion risks from presumably clean vessels arriving for extended layover in Baltic ports) or from other hard substrates in the high-risk areas (ports and marinas), e.g. underwater marine structures, navigational buoys or experimental settlement plates.

Biofilm collection from underwater structures (like ship hulls and port infrastructure) is intended for use by divers and allows getting good quality biofilm samples, with negligible contamination. A modified protocol (as described e.g. in Zaiko et al. 2016, von Ammon et al. 2018, 2019) can be applied for obtaining biofilm samples from sampling substrates retrieved from water (e.g. experimental

settlement plates), as part of a routine bioinvasion monitoring or early detection of new incursions within a pathway hub.

Taxonomic expertise is not obligatory, but for laboratory sample processing experience of using molecular methods and availability of related laboratory equipment is required.

2.2 Time and area

Sampling of biofouling from ship hull should be conducted when ship is moored at a port. Sampling of biofouling from fouling plates and other hard substrates should be conducted during summer when the seasonal vegetation of mobile and sessile epifauna is at its highest. Deployment sites should be close to ships mooring sites, easily reachable and accessible from the piers. A minimum three sample replicates should be collected at each selected hard substrate. A chronological order of the sampling sites should be maintained for comparable results. Sampling sites in port areas should comply with health and safety policies and permitting regulations of the local authorities.

2.3 Monitoring procedure

2.3.1 Monitoring strategy

Before sampling from a ship hull (and other floating structures), it is recommended, that the level of biofouling (LOF) (Floerl et al. 2005) is assessed by divers during the initial underwater inspection by divers or using underwater camera/ROV. Only vessels with LOF 0 to 3 (biofilm level) to be selected for molecular sample collection. The LOF scale ranges from '0' to '5' (Table 1).

Rank*	Description	Visual estimate of fouling cover
0	No visible fouling. Hull entirely clean, no biofilm on visible submerged parts of the hull.	Nil
1	Slime fouling only. Submerged hull areas partially or entirely covered in biofilm, but absence of any macrofouling.	Nil
2	Light fouling. Hull covered in biofilm and 1–2 very small patches of macrofouling (only one taxon).	1–5 % of visible submerged surfaces
3	Considerable fouling. Presence of biofilm, and macrofouling still patchy but clearly visible and comprised of either one single or several different taxa.	6–15 % of visible submerged surfaces
4	Extensive fouling. Presence of biofilm and abundant fouling assemblages consisting of more than one taxon.	16–40 % of visible submerged surfaces
5	Very heavy fouling. Diverse assemblages covering most of visible hull surfaces.	41–100 % of visible submerged surfaces

*Source: Floerl et al. 2005.

The structures with LOF 0 to 3 (biofilm level) should be selected for molecular sample collection. The recommended minimum information on vessel performance characteristics and travel history to be collected includes vessel name and type, deadweight tonnage (DWT), length overall (LOA), average speed (knots), time since last out-of-water maintenance (days), time since antifouling treatment was last applied (days), typical operating route (set or itinerant), number of port calls over last 12 months, region of operation, arrival region (where the vessel arrived from).

For the analysis of the biofilm from experimental plates and other solid substrates (e.g. navigational buoys) the level of fouling also should not exceed LOF 3 (Floerl et al. 2005). The recommended minimum information is the of deployment (days) for experimental plates and time since last out-of-water maintenance for the navigational buoys.

2.3.2 Sampling methods and equipment

A modified syringe device adapted from Pochon et al. (2015) is recommended for sampling from the underwater structures (Figure 1). To assemble the device, a sterilized 50 ml syringe was trimmed to allow the inclusion of a cut-down sterilized sponge (Whirl-pak™, Speci-sponges™, Nasco, Salida, CA, USA). The sponge disks are inserted into syringes prior to sampling in laboratory conditions (wearing gloves, wiping the working surfaces and scissors with DNA decontamination solution) and sealed with a sterilized rubber plug secured with the rubber band attached to the top of the syringe barrel. The assembled sponges and syringes are treated with UV light for at least 20 min and placed unplugged into a clean container until used underwater. Syringes can be re-used, ensuring thorough cleaning and bleaching after each sampling and re-inserting sterile sponges as described above.

For ex-water sampling from, sterilized sponges or sterile stainless-steel surgical blades can be used (see details in Zaiko et al. 2016, von Ammon et al. 2018, 2019), depending on type and stage of the biofouling material.

Materials required for underwater sampling:

- Modified sampling syringes (see Fig. 1)
- Magnet frame
- Chilly bin with ice (collected samples should be kept cold until delivered to the lab)
- Sampling bags (one for unused syringes, second – for syringes with collected samples)
- Individual sealing plastic bags for syringes.

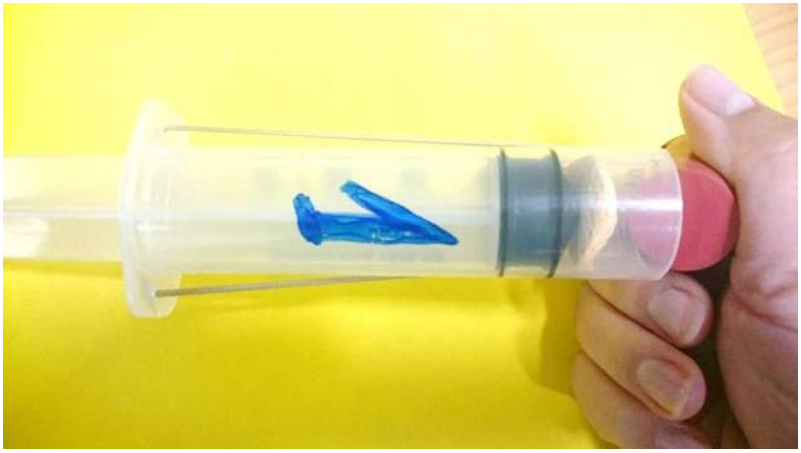

Figure 1: Modified syringe sampling device (photo: A. Zaiko)

Sampling procedure:

1. The magnetic frame is attached to the hull

2. The syringe is drawn from the bag, carefully unplugged, put against the hull at the sampling area (within the frame), the syringe plunger is pushed to expose the cut-down sponge and the biofilm collected by swiping the device (Fig. 2A) with three strong strokes across the sampling area

3. The plunger is pulled (while holding the syringe against the hull surface) and the syringe is immediately replugged

4. When landed, each syringe placed into individual sealing bags and hold on ice until delivered to the laboratory for further processing.

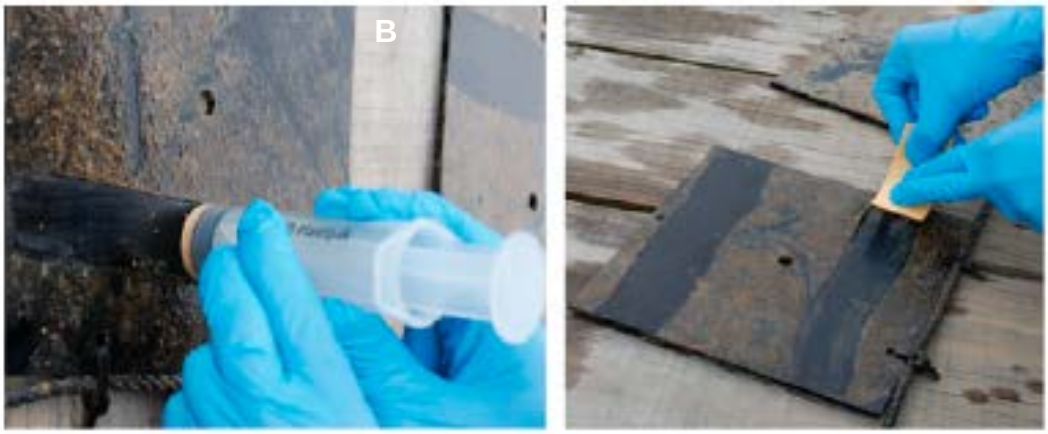

Figure 2: Biofilm collection with (A) the modified syringe device and (B) a sterilized sponge (Pochon et al. 2015).

For ex-water sampling, biofilm from a standardized area (e.g. a settlement plate) is swiped with a sterilized sponge (Fig. 2B) or scraped with a sterile blade and immediately isolated in a sterilized plastic bag (e.g. Whirl-pak™, Nasco) or sterile sample tubes respectively. Samples should be kept on ice until delivered to the lab for further processing.

If immediate processing of the samples is not feasible or practical, the samples can be stored frozen at -20°C.

2.3.3 Sample handling and analysis

For eDNA extraction from the sponges, RNA/DNA free water (40 ml) is added to the plastic bags, and the sponges are macerated with a laboratory stomacher for 2 min. Excess liquid is squeezed from the sponges applying consistent pressure and transferred to sterile 50 ml tubes. The suspension is pelleted by centrifugation (4000 × g, 15 min). The supernatant is discarded and eDNA is extracted from the pellet, following common protocols for DNA isolation from sediment or biofilm samples (for details see e.g. Zaiko et al. 2016). Biofilm material collected with a blade is directly processed for DNA extraction as described below.

There is a wide variety of DNA extraction kits available, and they are usually designed for a specific sample type (soil, water, biofilm, animal tissues, etc). We recommend the use of Qiagen™ PowerSoil kits for eDNA extraction from vessel biofouling samples, as Qiagen™ had successfully integrated its patented Inhibitor Removal Technology® and has proven to be effective for environmental DNA isolation from challenging samples such as enriched soils, plants and stools. Incorporating extraction blanks (i.e. controls) is required throughout the process, particularly when targeting bacterial communities, in order to determine any potential bacterial contamination of the DNA extraction kit and/or the reagents used (Salter et al. 2014). Finally, it is advised that the quality and purity of extracted products (including the controls) be verified using a Nano- or Spectro-photometer.

The DNA extracts 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 is confirmed by a validated targeted assay or visual assessment.

4. Contacts and references

4.1 Contact information

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4.2 References

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