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COMPLETE

# eDNA SAMPLING PROTOCOL FOR HULL BIOFOULING AND BALLAST WATER MONITORING, AND GUIDELINES FOR THE MONITORING OF TARGET NON-INDIGENOUS SPECIES USING MOLECULAR METHODS

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# CONTENT

# INTRODUCTION

This report has been developed as a part of the COMPLETE project (Completing management options in the Baltic Sea Region to reduce risk of invasive species introduction by shipping) funded by the Interreg Baltic Sea Region Programme. In the COMPLETE project, Klaipeda university has lead the development of the innovative tool-kit for detection of non-indigenous species (NIS), including potential harmful aquatic organisms and pathogens (HAOPs) using rapidly evolving molecular methods. This report presents the following parts:

1) guidelines for the monitoring of non-indigenous species in biofouling,

2) guidelines for the monitoring of non-indigenous species in ballast water of ships; and

3) guidelines for the monitoring of target non-indigenous species.

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. Applying the so-called environmental DNA (eDNA) -methods for water sampling leads to a higher probability detecting target species, comparing to e.g. sampling from sediments or settlement plates, and in combination with the traditional surveillance methods increases efficacy of target species detection. 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.

Molecular tools have been proposed for future HELCOM NIS monitoring guidelines at the 12$^{th}$ Meeting of the Working Group on the State of the Environment and Nature Conservation on 11-15 May 2020 (meeting documents available here), although it has been noted that further research and development is recommended to improve the representativeness of sampling, a better understanding of the durability of eDNA and eRNA in marine and ballast water, and the accumulation of bar codes for target invasive marine species in global reference libraries.

# PART 1. GUIDELINES FOR THE MONITORING OF NON-INDIGENOUS SPECIES IN BIOFOULING, WHICH ARE ACCESSIBLE BY MOLECULAR METHODS

## 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

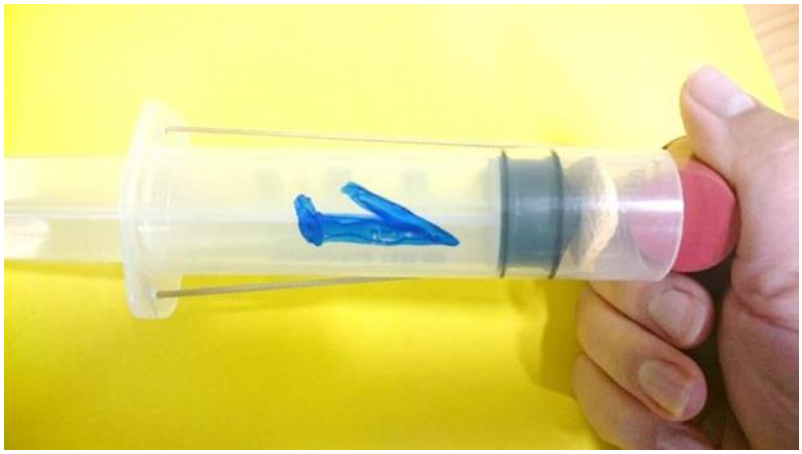

Figure 1: Modified syringe sampling device (photo: A. Zaiko)

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 QiagenTM PowerSoil kits for eDNA extraction from vessel biofouling samples, as QiagenTM 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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# PART 2. GUIDELINES FOR THE MONITORING OF NON-INDIGENOUS SPECIES IN BALLAST WATER OF SHIPS, WHICH ARE ACCESSIBLE BY MOLECULAR METHODS

## 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 ZRDuetTM 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

Anastasija Zaiko (anastasija.zaiko@jmtc.ku.lt)

## 4.2 References

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# PART 3. GUIDELINES FOR THE MONITORING OF TARGET NON-INDIGENOUS SPECIES USING MOLECULAR METHODS

## 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 <http://jointbwmexemptions.org/ballast_water_RA/apex/f?p=104:4>.

# 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

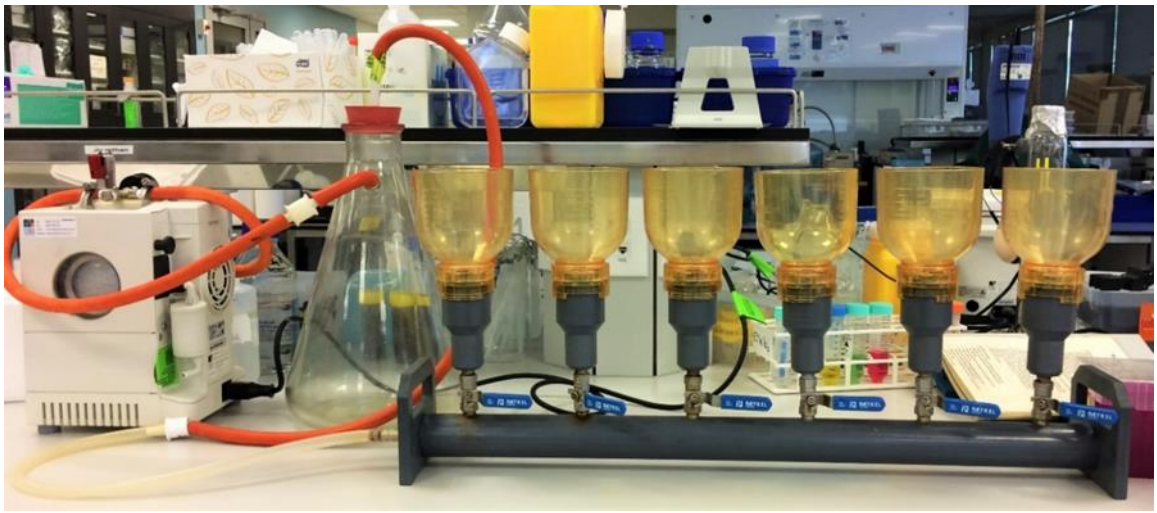

Figure 1: Six-way filtering system for lab-based filtering of water samples (photo: A. Zaiko).

## 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 to use 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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