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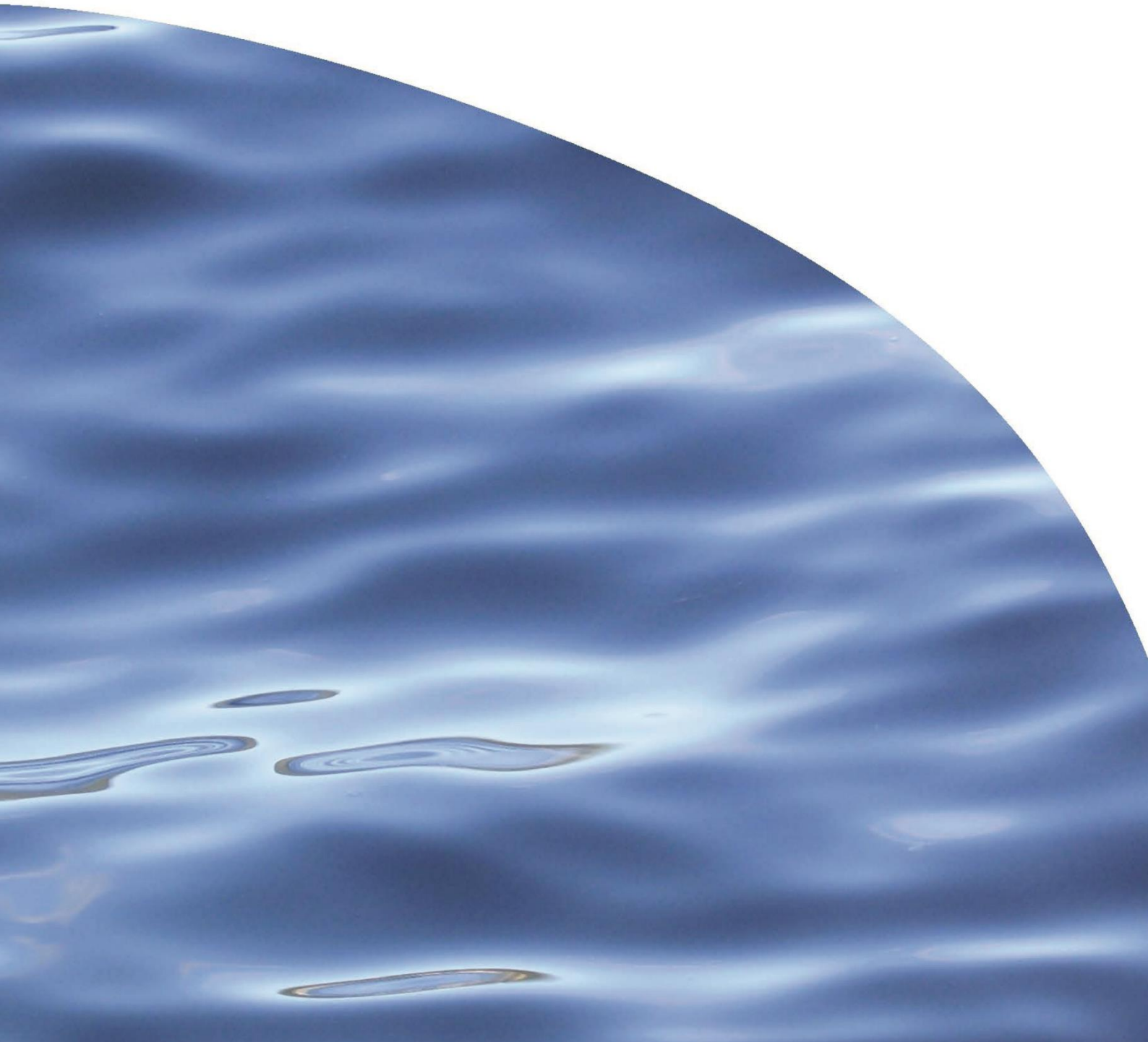

ENVIRONMENTAL DNA SCREENING OF *SABELLA SPALLANZANII* AND *STYELA CLAVA* FROM WATER SAMPLES IN THE TUTUKAKA MARINA

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1. INTRODUCTION

1.1. Background

The Tutukaka Marina is currently under a 5-year Mediterranean fanworm (*Sabella spallanzanii*) eradication program, in response to the detection of two small individuals in 2015. As part of a collaboration between the Ministry for Primary Industries (MPI) and Northland Regional Council (NRC), annual searches of structures and visual checks of hulls are being conducted. While diver searches remain the primary method for detecting *S. spallanzanii*, this method is costly and time-consuming and therefore cannot be deployed over entire habitats. Consequently, the risk of missing population 'pockets' might undermine recent eradication efforts. Confidence in the successful eradication of *S. spallanzanii* can be increased by using complementary molecular methods that combine the use of environmental DNA (eDNA) with species-specific Polymerase Chain Reaction (PCR[1]) techniques.

Environmental DNA is released into aquatic systems from a wide range of organisms, including plants, animals and microbes. It is contained within or released from multiple sources such as skin flakes, reproductive structures (sperm/eggs) and body fluids (urine, faeces). Once in the water column, DNA decays within days and therefore provides information on current diversity. Molecular techniques can be used to analyse eDNA and provide information on which taxa are present in a system. These techniques can detect any life stage including microscopic propagules, therefore have great potential for targeted species detection and biodiversity assessments at extensive spatial, taxonomic, and temporal scales.

In recent years, multiple New Zealand-based research projects applied real-time PCR assays to detect *S. spallanzanii*, as well as the clubbed tunicate, *Styela clava*, in environmental samples (Audrezet 2018; von Ammon et al. 2018; Wood et al. 2018, 2019; Zaiko et al. 2018). These studies indicate that the application of such techniques on eDNA filtrates from water samples can result in higher detection rates compared to sediments or biofilms.

Droplet digital PCR (ddPCR) is a recent advancement for quantitatively detecting targets in samples. The method partitions the samples into many thousands of reaction compartments (droplets) via microfluidics, reducing the competition from background DNA and providing high sensitivity. The compartments containing the target DNA are amplified via PCR, and individually screened via fluorescence measurement for the presence of target DNA. This approach negates the need to use standard curves and enables extremely low-level detection and considerably higher

[1] A method of producing multiple copies of a DNA sequence and involving repeated reactions with polymerase enzymes. The method uses primers, or primers and a probe that have been designed to be specific for the target species. The amplification of this target can then be measured in real-time either using intercalating dyes or probe-based detection systems.

cost-efficiency compared to real-time PCR. The droplets with the target gene present are counted as positive reactions, whilst those without the gene of interest are counted as negative reactions. Droplet digital PCR uses a Poisson distribution analysis derived from the number of positive and negative reactions to provide absolute quantification of the target DNA in the original sample. Droplet digital PCR is considered a robust, precise and sensitive tool for detecting and quantifying low-prevalence target nucleic acids (Hindson et al. 2011) and outperforms real-time PCR methods in detection of non-indigenous species (Doi et al. 2015; Wood et al. 2019).

Although molecular methods targeting eDNA are highly sensitive and provide excellent screening tools for species distributions across large spatial scales, a positive eDNA signal does not allow the exact location of the target species to be determined. This is especially the case for signals reported in water samples from coastal environments where currents and tides may transport eDNA over many kilometres. 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.

1.2. Scope of the study

The main objective of the present study was to provide NRC with an additional molecular method to increase confidence in the successful eradication of *S. spallanzanii* in 4 areas spanning the Tutukaka Marina and adjacent bay. This was achieved by collecting water samples and analysing these using droplet digital PCR (ddPCR). To maximise the output, a simultaneous test for the presence of *Styela clava* was also undertaken on the same samples.

2. METHODS

2.1. Field sampling and sample filtration

Water samples for eDNA analyses were collected on 18 November 2019 by NRC staff using plankton net tows. Collection followed the detailed protocols and guidelines provided by the Cawthron Institute (Appendix 1) and was undertaken from four areas within the Tutukaka Marina and the adjacent bay. At each area five sites were sampled with three replicated plankton net tows taken at each site (n = 60, see Figure 1 and Supplementary 1 for detailed information on the sampling design). Field blank (FB) controls were also randomly collected from three sites across the sampled areas.

These controls consisted of sample bottles filled with tap water, taken into the field, dipped into the sea and stored with actual samples during transport. The purpose of these controls was to ensure that no cross-contamination occured during sampling and sample handling in the field.

Concentrated plankton samples were filtered the next day by NRC staff after being refrigerated overnight and followed the protocol detailed in Appendix 2. Three 'blank filter' controls were included randomly throughout the filtration process to ensure that no laboratory-based cross-contamination occurred (laboratory blanks = LB). The filters were cut in half with clean scissors and both halves stored separately. Filters were sent on ice overnight to the Cawthron Institute for further processing.

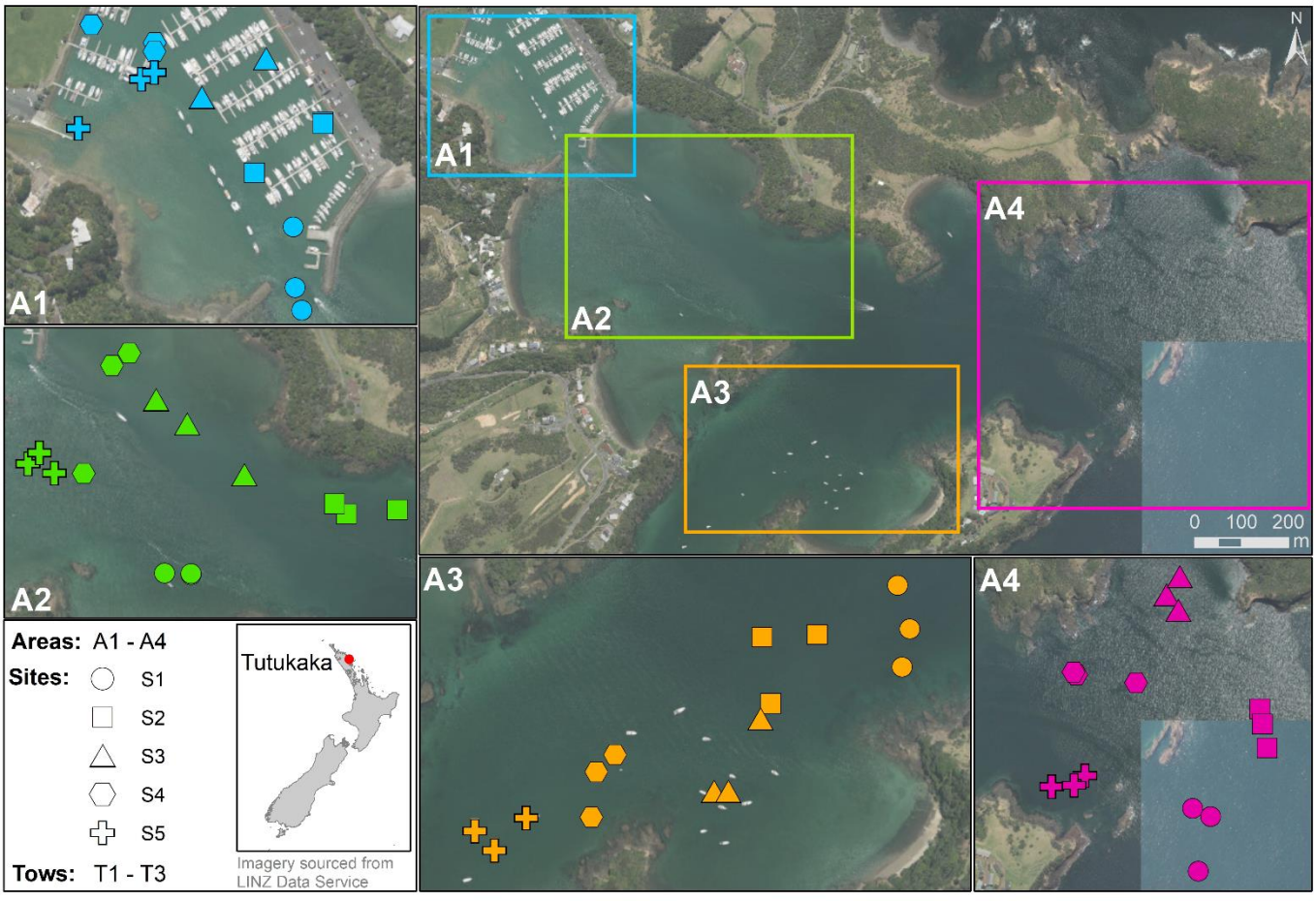

Figure 1. Sampling locations across 4 areas in the Tutukaka Marina and the adjacent bay, with 5 triplicate sampling sites per area

2.2. DNA extraction and droplet digital PCR

At the Cawthron Institute, one half of the filter was stored at -20˚C as a back-up, and the other half was processed for eDNA isolation using the PowerSoil DNA isolation kit (Qiagen, CA, USA). Extraction blanks (EB) were included to ensure no cross-contamination occurred during DNA extraction. The eDNA samples were then analysed using a QX200 Droplet Digital PCR System™ (BioRad), following the protocol described in Wood et al. (2018). All samples were run undiluted on one ddPCR plate including negative controls (no-template ddPCR control = RNA/DNA-free water Life Technologies™), and positive controls (genomic DNA extracted from *S. spallanzanii or S. clava* specimens) were included.

In case of ambiguous positive results (i.e. low copy numbers, close to the detection limit of 0.1 copies/µL; Wood et al. 2020), the relevant sample was re-run in triplicate to confirm or reject the positive detection with high confidence.

2.3. Occupancy modelling

To estimate *S. spallanzanii* occupancy (i.e. the likelihood of the target species to occupy the sampling locations), modelling based on a 'mark-recapture' approach is applied (MacKenzie et al. 2002). Occupancy estimation and modelling based on detection/non-detection data (detection histories) provides an effective way of assessing a species' distribution across time and space in cases where the species is not always detected with certainty (Nichols et al. 2008). Recently, site occupancy detection models, which provide an estimate of large-scale occupancy whilst accounting for imperfect detection, have been advocated as a way to overcome the potential issues of interpreting eDNA-based data during field surveys (imperfect detection, patchy distribution of eDNA in environment, and/or imperfect quantification; Lugg et al. 2017).

In the models, each sampling site represents a sampling unit within a focus area. Each replicated water sample (plankton net tow) taken at a site is considered an independent sampling occasion.The following parameters are defined in the models for considered species:

p_t = probability of detection at occasion *t,* given that sample unit is occupied, and the species is present at the immediate sampling site;

$\psi_{naïve}$ = naïve estimate of occupancy probability, calculated as the proportion of sample units where the species was detected over all units surveyed;

ψ = large-scale occupancy - probability of a sampling unit/area being occupied by the species.

3. RESULTS AND DISCUSSION

For both species, no positive ddPCR signals were detected in any of the sampling areas (eight ambiguous samples and four blank controls confirmed to be negative after re-run in triplicates, see Supplementary 2 for detailed results). No instances of cross-contamination were detected.

Given that we could not apply an occupancy model to the dataset due to no positive detections, we ran a simulation study as described by Guillera-Arroita et al. (2010), to explore probabilities of getting empty simulated detection histories (all non-detections) for the particular sampling design applied in this survey assuming low occupancy estimates if sparse *S. spallanzanii* populations are present in the region. The simulation parameters were:

- sampling design applied in this survey (with a primary focus on *S. spallanzanii* detection): total number of sampled locations S = 20 (4 areas x 5 sampling sites), K = 3 (3 replicates at each site), see Supplementary 1 for details;
- the detection probability is $\boldsymbol{p}$ = 0.7 (based on previous studies at locations with known *S. spallanzanii* populations, using ddPCR assay on water eDNA samples, see e.g. Wood et al. 2019);
- low expected probabilities of *S. spallanzanii* occupancy, $\boldsymbol{\psi}$ = 0.1.

The results showed that 6.7% of histories produced boundary estimates (i.e. overestimated occupancy $\boldsymbol{\psi}$ = 1 with low detection probabilities, see also detailed explanations in Supplementary 1), and 13% of generated histories resulted in empty histories (all zeros, when no detections happened despite species presence, Table 1).

Based on the rate of empty histories produced in this simulation, we can conclude that there is 13% probability of not detecting an established *S. spallanzanii* population with occupancy ψ = 0.1 (i.e. the species occupies 10% of the surveyed area).

Our recent experimental research has shown that *S. clava* releases substantially less eDNA in water compared to *S. spallanzanii*, most likely due to morphological peculiarities (Wood et al. 2020). This, consequently, affects the lower detection probabilities estimated for *S. clava* (p = 0.14, Wood et al. 2018). Therefore, the applied sampling design optimised for *S. spallanzanii* detection produced lower confidence results; i.e. 45.8% probability of not detecting established *S. clava* population with occupancy ψ = 0.1 (Table 2) and 13.7% probability of not detecting established *S. clava* population with occupancy ψ = 0.25 (Table 3).

Table 1. Results of the occupancy simulation for the sampling design applied in this study and assumed *Sabella spallanzanii* occupancy ψ = 0.1 (based on 10,000 histories of detection). The summary of the simulation results include: occupancy (psi) and detectability (p) estimator bias/variance/Mean Standard Error (MSE) (excluding empty histories - no detections); occupancy estimator bias/variance/MSE (excluding also boundary estimates, i.e. psi = 1); percentage of empty histories obtained; percentage of histories obtained that produce boundary estimates.

Evaluation of design K = 3 S = 20 (TS = 60), assumed occupancy ψ = 0.1 estimator performance (excl. empty histories)
ψ: bias = +0.0909 var = +0.0583 MSE = +0.0666 p: bias = -0.0328 var = +0.0663 MSE = +0.0673
estimator performance (excl. also histories leading to boundary estimates) ψ: bias = +0.0236 var = +0.0042 MSE = +0.0048 p: bias = -0.0210 var = +0.0341 MSE = +0.0345
empty histories = 13.0% boundary estimates = 6.7%

Table 2. Results of the occupancy simulation for the sampling design applied in this study and assumed *Styela clava* occupancy ψ = 0.1 (based on 10,000 generated histories of detection).

Evaluation of design K = 3 S = 20 (TS = 60), assumed occupancy ψ = 0.1 estimator performance (excl. empty histories)
ψ: bias = + 0.7107 var = +0.1344 MSE = +0.6395 p: bias = -0.0210 var = +0.0482 MSE = +0.0487
estimator performance (excl. also histories leading to boundary estimates) ψ: bias = +0.0074 var = +0.0059 MSE = +0.0059 p: bias = -0.3753 var = +0.0277 MSE = +0.1686
empty histories = 45.8% boundary estimates = 42.7%

Table 3. Results of the occupancy simulation for the sampling design applied in this study and assumed *Styela clava* occupancy ψ =0.25 (based on 10,000 generated histories of detection).

Evaluation of design K = 3 S = 20 (TS = 60), assumed occupancy ψ = 0.25 estimator performance (excl. empty histories)		
ψ: bias = + 0.5091	var = +0.1385	MSE = +0.3977
p: bias = -0.0064	var = +0.0361	MSE = +0.0361
estimator performance (excl. also histories leading to boundary estimates)		
ψ: bias = +0.0383	var = +0.0216	MSE = +0.0231
p: bias = +0.2433	var = +0.0274	MSE = +0.0866
empty histories = 13.7%		
boundary estimates = 59.9%		

The results of the current study provide reasonably high confidence of no established *S. spallanzanii* populations in the surveyed areas. Nevertheless, the presence of individual *S. spallanzanii* specimens within the study region cannot be completely excluded. Additionally, based on the lower confidence results for *S. clava,* we cannot exclude the presence of localized/sparse populations of this species in the area.

Based on these results, we recommend:

(i) Supplementing targeted marine biosecurity surveillance programmes in the Northland region with ddPCR assays using eDNA extracted from water samples to support optimisation surveillance strategies and allow early detection of S. spallanzanii *in otherwise unsurveyed areas, over extended spatial scale;*

(ii) Explore alternative methods for isolating eDNA from water to optimise and simplify sample collection;

(iii) Develop and apply fit-for-purpose sampling strategies for eDNA-based surveillance, taking into account harbour size, hydrodynamic peculiarities (exposure, tidal regime), proximity to introduction pathways and the history and distribution of the target species. Such a strategy would allow the determination of likely benthic source populations of eDNA signals detected form water samples;

(iv) For higher confidence of eDNA-based surveillance of S. clava*, the sampling design should be adjusted to account for lower eDNA shedding rates and detection probabilities compared to* S. spallanzanii*;*

(v) For absolute confirmation of species detection, it is recommended that positive signals from eDNA samples are followed up by visual searches.

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6. APPENDICES

Appendix 1. Plankton tow collection kit and protocol for molecular sampling as part of the *Sabella spallanzanii* detection from water eDNA samples.

Material needed (sampling)

✓ 2 plankton nets with 20 µm mesh size complete with ballasted collector (Fig. 1)

✓ 1.5 kg lead ballast attached to one end of a 5-6 mm dia. rope ca. 1 m long (Fig. 2)

✓ Float attached to one end of the 1 m line above (Fig. 2)

✓ Pulling line 15 m long (5-6 mm dia. rope)

✓ Sterile sample collection bottles (400 ml)

✓ Labels for collection bottles

✓ Squeeze/wash bottle (Fig. 3)

✓ Timer

✓ Two large (20 L) buckets for disinfecting/rinsing net, funnel, and other material. One bucket will be used for bleaching the gear, the other as a backup

✓ 3 L of bleach

✓ 1 box of sterile gloves

✓ Rubbish bag (for disposing gloves)

✓ Chilly bin(s) and ice/ pre-frozen ice packs (make sure that all sample bottles are fitting in)

✓ Field log sheets, field sampling protocol, site maps on water proof paper

✓ GPS device

✓ If possible, probe for measuring temperature, pH, conductivity

✓ Marker pen, pencil, pens, scissors

✓ Emergency net repair kit

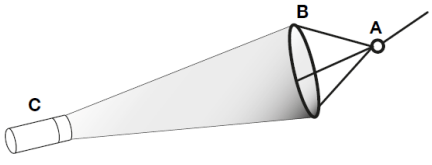

Fig. 1 - Plankton sampling net. A) central stainless steel ring, B) entrance ring, C) Collector bucket.

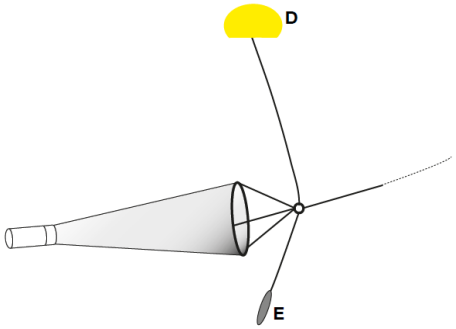

Fig. 2 – Scheme of the sampling setup. D) buoy, E) lead ballast weight.

Fig. 3 – Squeeze wash bottle

Before Sampling

- Label sample collection bottles and prepare field log sheets.
- Prepare 3 bottles for 'field sampling controls': fill sample bottles with tap water, label correspondingly (e.g. "field blank #1", etc.).
- Prepare chilly bin and ice packs for storing collected samples in the field.

Plankton Sampling

- Before leaving the port, fill 3/4 of the bucket with sea water. Fill a spare sample bottle with bleach (approx. 400 mL) and add to each bucket of water to make a ~2% solution. Put all sampling material (net, ballasted collector bucket (cod-end, removed from net) and the squeeze bottle) in the bleach bucket.
- At the first sampling site (first sampling area), put on gloves (please wear new gloves in every sampling area when touching/operating the gear), remove the net, cod-end and squeeze bottle from the bleach bucket, rinse thoroughly with seawater from the site. Fill in the squeeze bottle with the sea water from the sampling site.
- Place the second set of gear into the bleached bucket (will be used in the sampling area #2).
- Assemble the plankton net set up as in Figure 2: screw the collector bucket to the plankton net; the plankton net+ collector, the pulling line, the buoy and lead ballast weight are all connected to the same carabiner. Make sure that the end of the pulling line is securely fixed to your boat to prevent losing the net.
- Reduce speed of the boat to a maximum of 2 knots and maintain this throughout the towing. Lower the net into water and let all the air out. Start unrolling the pulling line such that it always remains under tension and simultaneously register in the log sheet the GPS position, starting time, boat's speed relative to water, and course.
- Make sure the tow stays at least 1 m depth. Towing time is 1 minute. Tow times can be reduced to 30 seconds if a lot of organic material impede the filtering of the sample as described in the next step. Take note in the field log form if tow times are changed.
- After 1 min gently recover the pulling line — keeping it always under tension — and, as soon as the net is out of water, record GPS position and ending time in the log sheet.
- Once on board, rinse the walls of the net using the water from the squeeze/wash bottle which needs to be filled with sea water from the sampling site and let all the particles fall into the collector. Pour out some of the collected liquid through the net mesh to adjust its level just below the collector's screw, otherwise part of the sample will overspill when unscrewing the collector.
- Dismount the ballasted collector bucket and transfer its content (~400 mL) into the labelled sample bottle. Close the bottle with the lid and put on ice.
- Repeat 3 times (3 tows) at each pre-defined sampling site. Before moving to another sampling site, discard the seawater from the squeeze bottle.
- At the next sampling site (within the same sampling area), rinse thoroughly the

net and the collector with seawater from the site. Fill in the squeeze bottle with the sea water from the sampling site repeat the procedure as described above (starting “Plankton sampling”, bullet point #3).

- When moved to the next sampling area, remove the second set of gear (net, collector, squeeze bottle) from the bleach bucket and repeat the procedure as described above (starting “Plankton sampling”, bullet point #2). Place the first set of gear into bleach bucket until the next sampling area.
- ‘Sampling controls’ – at three different sites (it doesn’t matter which) immerse the control bottles in the sea (leaving lids closed) and place back in chilly bin with other samples. Take note in field log sheet if and which (#1,2,3) ’field sampling controls’ have been done.

Appendix 2. Water filter collection kit and protocol for molecular sampling as part of the Mediterranean flatworm *Sabella spallanzanii* detection from water eDNA

Material needed (lab filtration)

✓ 3 sets of tweezers
✓ Scissors
✓ 1 box of sterile gloves
✓ 1 box of 100 glass microfiber Whatman filters, grade GF/C (1.2 µm pore size, 47 mm dia.)
✓ Filtration set (Fig. 1)
✓ Sterile tubes 1.7-2 ml. (At least 2 per sample, can be up to 10 per sample)
✓ freezer boxes for tubes
✓ 20 L bucket
✓ Paper towels/paper tissue
✓ 2% bleach solution
✓ 20 L bucket
✓ safety glasses
✓ filter log sheet
✓ tap water for 'lab control blanks' (400ml x 3)

Fig. 1 - Filtering system for lab-based filtering of water samples. Three-fold manifold (magnetic system).

Before filtration

- Prepare filter log sheet and familiarise yourself with filtration unit (*Fig. 1*).
- Label ~2ml tubes indicating sampling area, sampling site, replicate and subsample (optional, if more than 1 filter used per sample) – e.g. A1.2-a corresponds to sampling area A, sampling site 1, replicate 2, subsample a.
- Label freezer boxes indicating project client and project (sampling event) number (e.g. NRC1) and filter half (1 or 2) – NRC1 – filter half 1
- Set up one 20L bucket with 2% bleach (fill ~3/4 and add ~300ml of Bleach)
-

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.

- Put on gloves and clean working surfaces (i.e. laboratory bench) with 2% bleach solution.
- Soak the funnels and filter holders of the filtration unit, tweezers and scissors in 2% bleach solution for at least 20 min, rinse well with tap water and dry on a clean paper towel or dry with a clean paper tissue.
- Wear gloves and keep them on throughout the following procedure. Consider changing if samples spill on them or other contamination is suspected.
- Assemble the filtration unit without filtration funnels.
- With sterile tweezers take a glass microfiber Whatman filter and place it on the support screen of the filter holder, making sure that it lies completely flat.
- Attach the funnel using the magnetic seal.
- Gently shake the sample bottle to ensure its homogeneity and gradually pour one half (~200 mL) of it in the funnel.
- Turn on the vacuum pump until the liquid is completely filtered and collected in the receiver flask. Pour the rest of the sample and filter as before until no water is visible on the filter, keep pumping for approximately 30 sec to dry the filter.
- Remove the filtration funnel. With one tweezer holding the filter (not fully removing the filter from the holding screen), cut 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). Place the tubes in the freezer box (keep cold). Place all ½ filters from all sites in one freezer box (labelled with Filter half 1, these will be processed) and the other ½ from all sites in another freezer box (labelled with Filter half 2, these will be stored as back up).
- Ensure tubes are labelled properly and make sure the sampling site details, and volume filtered per filter as well as number of filters used per sample is recorded in the filter log sheet.
- Bleach and wash all material in between Sample sites and areas to avoid cross contamination. Scissors and tweezer may alternatively be sterilized with ethanol and flaming.
- Include three ‘Lab control blanks’: During the process of filtration, add any time using a freshly bleached set up, filter 400ml of tap water onto a filter as described above. Label with Bl1-3.
- When filtrations are complete, keep samples refrigerated and deliver for processing to Cawthron on ice overnight. If samples cannot be delivered within 24 hours after filtration, store them at -20˚C immediately after filtering.