

1 **Environmental DNA sampling protocols for the surveillance of marine non-indigenous species in**
2 **Irish coastal waters.**

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19 **Abstract**

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3 20 Understanding the spread and distribution of Non-Indigenous Species (NIS) is key when implementing
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5 21 legislation to maintain good ecosystem health. Environmental DNA (eDNA) has shown great potential
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7 22 to detect aquatic organisms in a rapid and cost-effective way, however their applicability to new
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9 23 environments must be validated prior to their implementation. Here, we tested different field
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11 24 sampling methods in combination with eDNA metabarcoding to develop a tool to detect NIS. Large
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13 25 and small volumes of seawater were filtered, in addition to the collection of sediment and horizontal
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15 26 tow net samples at 12 locations across four distinct geographic areas in Ireland. The biggest
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17 27 dissimilarity in the species recovered was found between sediment and town net samples. Tow nets
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19 28 showed to be the most efficient. A total of 357 taxa were identified, including 16 NIS. Fine mesh tow
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21 29 nets were identified as the most cost-efficient for large-scale monitoring and surveillance of NIS.
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27 30 **Keywords:** COI; eDNA; high throughput sequencing; rapid detection of species; non-indigenous
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29 31 species; metabarcoding.
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Highlights

- Marine NIS monitoring in Irish waters is crucial to detect invasive species at early stages of introduction.
- Highest number of NIS were found in Dublin and Carlingford sampling points as it could be expected for being high risk locations due to introduction vectors such as ships.
- Fine mesh tow nets were the most cost-efficient method for surveillance of NIS using eDNA metabarcoding.

32 Introduction

33 Non-Indigenous Species (NIS) are considered one of the main threats to global biodiversity (Mazor et
34 al., 2018). Several countries have recently adopted Maritime Spatial Planning (MSP) frameworks to
35 harness oceanic assets, boost maritime economies with sustainable practices, and preserve marine
36 habitat biodiversity (Directive 2014/89/EU) (<https://www.msp-platform.eu/countries-overview>). The
37 presence of NIS can negatively affect marine-based industries such as aquaculture and shipping, and
38 interagency efforts are ongoing to address specific sectoral impacts (Lacoursière-Roussel et al., 2018;
39 Molnar et al., 2018). Accordingly, NIS are being increasingly recognized in numerous regulations (e.g.
40 EC 708/2007; EC 1143/2014) as a key agent driving the loss of native species and are known to have
41 significant impacts on natural capital assets (Lovell et al., 2006; Mazor et al., 2018). The long-term
42 sustainability of marine ecosystems relies on the effective management of NIS (Lehtiniemi et al., 2015;
43 Lodge et al., 2006) and legislation [e.g. the EU Marine Strategy Framework Directive (MSFD) and the
44 Habitats Directive] indicates the need for monitoring the distribution and spread of NIS as a crucial
45 first step (Darling and Mahon, 2011; Duarte et al., 2020b). This stems from the evidence that early
46 detection and rapid eradication of NIS is fundamental. The use of sensitive monitoring tools increases
47 the rate of detection and successful eradication between introduction and establishment (Lodge et
48 al., 2006). To facilitate the implementation of regulations and management strategies, standardized
49 protocols are needed for *in situ* surveillance of marine NIS in both nearshore and offshore waters and
50 benthic habitats.

51 The isolation of DNA from environmental samples coupled with High-Throughput Sequencing (HTS)
52 technologies, known as eDNA metabarcoding, has emerged as a tool to unravel biodiversity patterns
53 and detect NIS (Ardura et al., 2020; Rey, Basurko, and Rodriguez-Ezpeleta, 2020; Brown et al., 2016;
54 Comtet et al., 2015; Holman et al., 2019). The amplification of DNA barcoding regions from
55 environmental samples such as sediment or water (eDNA metabarcoding) can determine the
56 incidence of taxa by comparing sequenced regions against reference databases (Creer et al., 2016;

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Duarte et al., 2020a). Existing survey methods for detecting marine NIS using net trawls and dredging are geographically limited, time consuming and can cause damage to ecosystems (Bohmann et al., 2014; Thomsen et al., 2016; Zou et al., 2020). The application of molecular surveys provides a cost-effective, sensitive, non-invasive, and rapid approach to characterize biodiversity in all seasons (Duarte et al., 2020b; Pochon et al., 2013; Thomsen et al., 2016; Yang and Zhang, 2020; Zinger et al., 2019). Several studies (e.g. Afzali et al., 2020; Closek et al., 2019; Deiner et al., 2017; Fernández et al., 2019; Olds et al., 2016) reported higher recovery of taxa captured with eDNA approaches than conventional counterparts; thus demonstrating that eDNA coupled with HTS is a more sensitive method for general biodiversity assessments in many scenarios. Utilizing early detection of NIS and rapid eradication surveillance strategies while concurrently complying with internationally adopted MSP, eDNA-based biodiversity monitoring provides the capacity to detect new NIS arrivals in real-time and map the current spread to help predict future expansion/contraction of established populations. Although many recent studies employing eDNA metabarcoding have recognised these benefits (e.g. Djurhuus et al., 2018; Holman et al., 2019; Jeunen et al., 2019; Koziol et al., 2019; Rey, Basurko, and Rodriguez-Ezpeleta, 2019), the specific field methodology chosen requires validation depending on the environment (Cristescu and Hebert, 2018), local communities and target taxa. Parameters such as salinity, UV exposure, and sediment composition, vary according to the geophysical location of the study, and can influence obtained sequence data (Collins et al., 2018). Depending on the species composition of the study site, sampling methodology can influence which species are recovered. For example, if a bulk sampling method is chosen, nontarget phytoplankton could assume a high read abundance percentage if abundant (Aylagas et al., 2016). Thus, sampling type is a key factor in reducing false negatives, as this will reduce the ability to pick up rare target taxa if fewer reads are available. Previous studies have concluded that a combination of sample types would be required to evaluate marine communities and detect NIS species (Holman et al., 2019; Koziol et al., 2018; Rey et al., 2019), however, it is time-consuming and costly to use multiple sampling types at each location. Target organisms in biodiversity studies span the entire taxonomic spectrum of living organisms

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83 occurring on all substrata (benthos, plankton, etc.). Furthermore, detectability may change across the
84 variable morphology of different life cycle stages, as is common for marine species with larval stages
85 prior to settlement and establishment as adults. With the aforementioned broad range of abiotic
86 parameters and vertical distribution of target species, it is necessary to conduct preliminary
87 methodological studies to enhance sensitivity of detection.

88 Here, we tested several methodological approaches combining different sampling strategies and
89 eDNA metabarcoding to determine the most effective strategy to detect marine NIS. The sampling
90 approaches included established protocols (1L of water filtered at the surface) in addition to novel
91 methods (High volume samplers), and were tested in locations with variable numbers of expected NIS
92 (e.g. ports, marinas, shores and open water) including areas previously identified as potential hotspots
93 of NIS by the risk assessment study by Tidbury et al (2016). The main aim of this study was to evaluate
94 rapid, practical, and cost-effective protocols for the monitoring and surveillance of marine NIS and
95 invasive species in Irish coastal waters.

96 **Materials and Methods**

97 **Study area**

98 Four main areas were chosen around the coast of Ireland, including Galway Bay, Waterford Estuary,
99 Dublin Bay and Carlingford Lough (Supplementary Figure 1). These were chosen to encompass a range
100 of sites from known/expected NIS hotspots (due to convergence of NIS pathways and vectors) to less
101 affected areas (e.g. Galway Bay) (Tidbury et al., 2016). Within each area, three sites were chosen to
102 encompass a range of environments affected by the primary NIS pathways (shipping and aquaculture),
103 including ports, marinas and open water locations (Supplementary Figure 1). Sites were chosen with
104 at least two meters of water depth at lowest tide and were either accessed by boat or directly from
105 fixed infrastructures (e.g. piers or floating docks). Physical-chemical parameters were measured using
106 a Horiba U-50 multimeter (Supplementary table 1).

107 **Field sampling**

108 Sampling took place between July and August 2019. At each location, we implemented four separate
109 field sampling methods: sediment cores, low-volume water filtration, horizontal tow net sampling,
110 and autonomous high-volume water filtration.

111 *Sediment samples*

112 Sediment samples were taken by deploying a box corer (150mm length, 150mm width and 170mm
113 height) and taking three samples (field repetitions) within each site. Approximately 10 mL of sediment
114 was removed from the top 5 cm layer using a sterile 50 mL falcon tube, which was subsequently
115 preserved with 3X volume silica beads and kept in the dark at ambient temperature. Field blanks were
116 taken at each site before deployment of the box corer by rising the device with 100% ethanol and the
117 ethanol was retained (approximately 50mL) for later DNA extraction.

118 *Low-volume water samples*

119 Surface low-volume (SLV) water was sampled by taking three 1 L samples using sterile bags (Whirl-
120 pak®, Nasco, WI, USA) either directly by hand or (when water surface was not easily accessible) using
121 a Niskin sampler. Deep low-volume (DLV) water was also collected (three 1 L samples as well) at depth
122 (approximately 1 meter above the sea floor) at each location using a Niskin sampler. All water samples
123 were filtered onsite using a peristaltic pump just after collection using 47mm diameter 0.45 µm
124 cellulose nitrate membranes preloaded into single-use sterile filter holders (Analytical Test Filter
125 Funnels, Thermo Scientific™ Nalgene™; Fisher scientific). Upon filtration each membrane was placed
126 in a sterile 15 mL tube and approximately 6 mL of silica beads were added to each tube for temporary
127 storage in the dark at ambient temperature prior to final storage at -20°C. Field blanks for water
128 samples were collected at each location and consisted of 1L of distilled water placed in the Niskin
129 sampler prior to being transferred to a Whirl pack bag and processed in the same manner as other
130 water samples.

131 *Tow net samples*

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3 132 Tow net samples were taken with a 41 µm mesh net (length of 100 cm and diameter of 40 cm) (Hydro-
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5 133 Bios, Kiel, Germany) pulled by hand along floating docks/piers or on a boat at a half knot for 50 m (for
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7 134 an estimated max volume of water filtered of up to 6.3 m³). Once towing was completed, excess water
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9 135 from the tow net was flushed through the side window of the cod end bucket (with the same mesh
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11 136 size as the net). The retained sample was recovered into 50mL tubes by rinsing the net and bucket
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13 137 with molecular grade (200 proof) absolute ethanol. If visible plankton mass was greater than 10 ml, a
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15 138 second falcon tube was used and topped with ethanol to ensure a final sample:ethanol ratio of at least
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17 139 1:5 (Taberlet et al., 2012). Tubes were kept in the dark and stored at -20°C upon arrival to the lab until
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19 140 further processing. Field blanks were taken before net deployment by rising the net with 100%
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21 141 ethanol and then collecting the ethanol to be analysed as a sample.
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27 142 *High-volume water samples*

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30 143 High-volume (HV) water samples were collected using a semi-automated Mark II inDepth eDNA
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32 144 sampler (Mynott and Marsh, 2020) (Applied Genomics, Brixham, UK), which was deployed using an
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34 145 anchor-rope-buoy mooring system to keep the sampler upright approximately 1m from the sea floor
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36 146 at each sampling location. The sampler allowed *in-situ* filtration of approximately 50 L of water over a
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38 147 25h period through a 1 µm polyethersulfone filter membrane (Effective Filtration Area 1300 cm²). The
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40 148 sampling algorithm operating the programmable pump ensures even sampling over this time period
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42 149 by progressively increasing sampling effort to compensate for reduced flow rate as suspended
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44 150 material progressively accumulates on the surface of the filter. The enclosed filter unit was removed
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46 151 from the sampling instrument, filled with fixative solution (Applied Genomics, Brixham, UK) and spiked
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48 152 with 1 mL of a synthetic DNA Internal Positive Control (Applied Genomics, Brixham, UK) to be used in
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50 153 downstream quality control steps. Samples were stored at room temperature away from any light
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52 154 source prior to further processing.
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155 All samples (water, sediment, and tow net) were taken in rapid succession within the 25 hours of
156 deployment of the HV sampler except for one site (Carlingford marina; Site 8) where sampling
157 occurred approximately 12hrs post collection of the HV sampler due to adverse weather conditions.

158 All non-disposable sampling equipment (i.e. box corer, niskin samplers, tow nets, high-volume water
159 samplers, anchors, ropes, and buoys required for deployment) was decontaminated with a sodium
160 hypochlorite solution at a concentration of 10,000 parts per million available chlorine (20% dilution of
161 a general-purpose commercial bleach; product code A065EEV2 - Evans Vanodine International plc)
162 prior to visiting each sampling site. To avoid potential contamination during transport, all equipment
163 was kept enclosed in clean bags and handled with gloves.

164 **DNA isolation**

165 DNA was extracted from sediment samples using DNeasy PowerMax Soil kit (Qiagen, Hilden, Germany)
166 and following manufacturer's instructions using 5 g of sample as starting material. The same kit was
167 used to extract DNA from HV samples, extractions from HV samples were developed by Applied
168 Genomics following manufacturer's recommendations with additional modifications to accommodate
169 for pooling of large starting volumes (further details available at Applied Genomics, Brixham, UK).

170 For filter membranes, DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden,
171 Germany) following manufacturer's instructions on "Purification of Total DNA from Animal Tissues
172 (Spin-Column Protocol)", with the following modifications. Each filter membrane was cut in half with
173 scissors and placed in a 10mL tube containing 0.25 g of 0.1 mm glass beads and 0.25 g of 0.5 mm glass
174 beads (Oakton™ Glass Beads for Mills). Both scissors and tweezers used for cutting and handling the
175 membranes were decontaminated with Dna exitus plus™ prior to use. An initial bead beating
176 modification step was incorporated for 15 min at half speed using the mixer mill MM 400 homogenizer
177 by Retsch (Haan, Germany). 720 µL of ATL Buffer, 950 µL of distilled water and 100 µL of Proteinase K
178 (2 mg/L final concentration) were added in place of the recommended volumes. The final elution step
179 was carried out by flushing 100µl elution buffer AE through the column twice for a final total volume

180 of 200 μ L. The same extraction protocol was used to extract DNA from tow net samples, with the
181 starting material generated by pelleting 15 mL of the sample, removing the supernatant, and adding
182 1350 μ L of ATL Buffer.

183 Extracted eDNA was quantified with a Qubit[®] 3.0 fluorometer using the High Sensitivity double
184 stranded DNA (HS dsDNA) reagents kit (Invitrogen Carlsbad, CA, USA). An extraction negative control
185 was included on each DNA extraction round and processed alongside samples throughout all
186 subsequent steps. Field negative controls were also extracted following the pipeline of each
187 technique. All extractions were performed in a pre-PCR room under a flow laminar hood with UV
188 decontamination.

189 A further purification of the nucleic acids was conducted using an OneStep PCR Inhibitor Removal Kit
190 (Zymo Research, D6030) according to manufacturer's instructions using 100 μ L volume of the sample.

191 **Mock communities**

192 A total of 20 species spanning a range of taxonomic groups were chosen to create five mock
193 communities to be used as controls to assess the effectiveness of the metabarcoding pipeline. These
194 included species belonging to Mollusca (*Pecten maximus*, *Crassostrea gigas*, *Ensis siliqua*, and *Mytilus*
195 *edulis*), Annelida (*Sabellaria alveolata*, *Arenicola marina*, and *Ficopomatus enigmaticus*), Chordata
196 (*Styela clava* and *Botryllus schlosseri*), Arthropoda (*Amphibalanus improvisus*, *Amphibalanus*
197 *amphitrite*, *Caprella mutica*, *Homarus gammarus*, and *Carcinus maenas*), Bryozoa (*Bugula neritina* and
198 *Watersipora subatra*), Ochrophyta (*Thalassiosira weisspogii*), Rhodophyta (*Schizymenia apoda*), and
199 Cnidaria (*Cordylophora caspia* and *Diadumene lineata*).

200 Mock community 1 featured an equal proportion of all 20 species at the same concentration (0.5
201 ng/ μ L), while Mock communities 2 through 5 were comprised of varying concentrations of the 20
202 species, see %expected in Supplementary table 2.

203 To check if there was a correlation between the expected versus the observed percentage of reads
204 belonging to each of the OTUs included in the five mock communities (Klymus et al., 2017), a Pearson
205 correlation test was performed using excel software version 2016. Only reads that were an exact
206 match to genus level were retained (except for *Conticribra weissflogii* that belongs to the same family
207 than *Thalassiosira weisspogii* and are commonly misidentified).

208 **eDNA amplification using high throughput sequencing**

209 *Inhibition testing*

210 To check for potential presence of enzymatic inhibitors in the DNA extracts, a quantitative Polymerase
211 Chain Reaction (qPCR) assay targeting an internal positive control was carried out using Applied
212 Biosystems® TaqMan® Exogenous Internal Positive Control Reagents (Thermo Fisher Scientific,
213 Waltham, MA, USA). Amplification (C_t) values were compared between reactions containing 2 μ L PCR
214 grade water and reactions containing 2 μ L of template eDNA. To enable an appropriate assessment of
215 potential inhibition during subsequent steps, Invitrogen™ Platinum™ II Hot-Start PCR Master Mix (2X)
216 was used in the qPCR inhibition testing as in the subsequent HTS steps (i.e. amplicon generation and
217 library preparation). Each qPCR was carried out in a 25 μ L reaction volume containing, 2.5 μ L of 10X
218 Exo IPC Mix and 0.5 μ L of 50X Exo IPC DNA. The thermal cycle profile consisted of a hot start at 94°C
219 for 2 minutes, a denaturing step at 98°C for 5 seconds, and an annealing step at 60°C for 15 seconds
220 for 45 cycles. Two technical replicates were performed for each sample and qPCR negative controls
221 were included in each run.

222 *Library preparation and HTS*

223 For amplicon generation and library preparation, a 313 bp portion of the Cytochrome c Oxidase
224 subunit I (COI) mitochondrial DNA gene was amplified using primers mICOLintF-XT (5'
225 GGWACWRGWTGRACWNTNTAYCCYCC 3') and jgHCO2198 (5' TANACYTCNGGRTGNCCRAARAAYCA

226 3')(Wangenstein et al., 2018), modified from Leray et al. (2013). These primers contained an adapter
227 sequence used as a target for the second PCR.

228 For each sample, PCR was carried out in triplicate in a final volume of 20 μ L, containing 1 μ L of template
229 DNA (ranging from 0.2 to 145 ng/ μ L), 0.4 μ M of each primer, 1X Platinum™ II Hot-Start PCR Master
230 Mix (Thermo Fisher Scientific), and ultrapure water up to 20 μ L. The reaction's thermal profile was as
231 follows: an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 47 °C for 60
232 s, 68 °C for 60 s, and a final extension step at 68 °C for 10 min. Negative controls that contained no
233 DNA (BPCR) were included in every PCR round to check for contamination during library preparation.
234 The PCR products were run on 2 % agarose gels stained with GreenSafe (NZYTech) and imaged under
235 UV light to verify the amplicon size. The three PCR replicates were pooled together in order to make
236 one single library from each sample. The oligonucleotide indices which are required for multiplexing
237 different libraries in the same sequencing pool were attached to the pooled triplicates in a second PCR
238 round with identical conditions but only 5 cycles and 60 °C as the annealing temperature. For a
239 schematic overview of the library preparation process, please see Figure 1 in Vierna et al. (2017). The
240 libraries were run on 2 % agarose gels stained with GreenSafe (NZYTech) and imaged under UV light
241 to verify the library size. Libraries were purified using the Mag-Bind RXNPure Plus magnetic beads
242 (Omega Biotek), following manufacturer's instructions. Then, libraries were pooled in equimolar
243 amounts following quantification using a Qubit dsDNA HS Assay (Thermo Fisher Scientific). This pool
244 also contained 10 μ L of each of the negative controls (both the extraction negative controls and the
245 PCR negative controls -BPCR-). The pool was sequenced in a NovaSeq using PE250 kit (Illumina). Library
246 preparation and HTS services were carried out by AllGenetics & Biology SL (www.allgenetics.eu).

247 **Bioinformatics**

248 Illumina Paired-End raw files consisting of forward (R1) and reverse (R2) reads were sorted
249 (demultiplexed) by library and quality scores. Indices and sequencing primers were trimmed during

250 the demultiplexing step. The raw FASTQ files can be accessed at NCBI SRA file archive PRJNA678844
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5 252 The quality of the FASTQ files was checked using the software FastQC (Andrews, 2010). R wrapper
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7 253 JAMP v0.67 (<https://github.com/VascoElbrecht/JAMP>) was employed to quality filter the data:
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10 254 sequences were merged using Usearch v11.0.667_i86 (Edgar, 2010) allowing for a 25% mismatch in
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12 255 overlap (setting “-fastq_maxdiffs 99 -fastq_pctid 75 -fastq_trunctail 0” as thresholds). Primers were
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14 256 trimmed and sequences were filtered by length (amplicon size 313 ±10 bp reads were retained) using
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17 257 Cutadapt v1.15 (Martin, 2011). Expected errors probabilities were calculated to filter reads qualities
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19 258 (Max ee=0.5; -fastq_qmax 60) using Usearch v11.0.667_i86 (Edgar, 2010). Denoising option was
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22 259 employed within JAMP pipeline, where sequences are dereplicated and denoised using
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24 260 the unoise3 algorithm from Usearch v11.0.667_i86 (Edgar, 2010) and clustered at 97% similarity.
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27 261 Then, OTU’s below 0.01% abundance are discarded, as well as haplotypes below 0.003%.

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30 262 For taxonomic classification, filtered sequences were compared against a public COI reference Nt
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32 263 database (NCBI, accessed on 16/06/2020) and stored locally. The database was downloaded using the
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34 264 esearch query “COI NOT Bacteria NOT environmental NOT viruses NOT unclassified” and constructed
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37 265 with the respective taxonomic information using the script “Entrez_qiime.py” by Baker (2017). Finally,
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39 266 “qiime feature-classifier” command within QIIME™ 2 pipeline v2020.2 (Bolyen et al., 2019) was
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42 267 employed to assign the taxonomy, using a 97% as identity percentage threshold and an e-value of 10⁻⁵⁰.
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47 269 Resulting Operational Taxonomic Units (OTUs) from taxonomic assignment were edited and
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50 270 statistically analysed using Microsoft Excel version 2016.

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52 271 Non indigenous species names were checked using the World Register of Introduced Marine Species
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55 272 (Ahyong et al., 2021) and their status (i.e. native or introduced) scrutinized in both global and regional
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58 273 databases (i.e Minchin 2007, Ahyong et al., 2020).

274 **Statistics**

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3 275 Statistical analyses were performed using R software v3.6.2 (R Core Team, 2020). Upset plots were
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5 276 constructed to show the overlap on the number of species recovered using “UpsetR” package (Conway
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8 277 et al., 2017). Read depths were adjusted to the same minimum depth using *Subset* function within
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10 278 JAMP pipeline prior to perform the statistical analyses. Shapiro-Wilk normality tests were performed
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12 279 to check number of OTUs and NIS distribution. A permutational multivariate analysis of variance
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14 280 (PERMANOVA) was used to test the influence of the factors “Method”, “Location” and “Site type (Port,
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17 281 Marina or Open Waters)” on the number of OTUs, using *adonis2* function with “jaccard” method and
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19 282 999 permutations within “vegan” package, *betadisper* function was performed to check the dispersion
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22 283 of the data (Supplementary table 3). Kruskal Wallis tests were performed to check if the influence of
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24 284 the Method in both the number of OTUs and NIS detected was significant. Ad-hoc pairwise
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26 285 comparisons using Wilcoxon rank sum test were later performed to check what levels were giving the
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29 286 significance to the factors if any significance was found.

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32 287 **Results**

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35 288 A total of 99,391,252 reads were obtained as raw output from sequencing. 23,567,217 (48.61%) reads
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37 289 were obtained after merging and quality filtering and from those, 2,240,625 (9.51%) were
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40 290 taxonomically assigned (Supplementary table 2). Evidence of contamination was found in some
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42 291 negative controls (Supplementary table 4). OTUs found in those controls were discarded from the
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44 292 analyses with the exception of NIS species that were deleted for statistical analyses but kept in NIS
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46 293 detection report if the number of reads found in the sample was higher than the number of reads
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49 294 found in the negative controls per site.

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52 295 *Mock communities*

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55 296 97% of the 349,699 reads recovered from mock community samples matched sequence data from the
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58 297 target species. Only OTUs resolved beyond the genus level were retained for the analyses and 3% of
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298 total reads (i.e. matching species not included in the original mock) were discarded. Reads originating
299 from *Thalassiosira weisspogii* were erroneously assigned to *Conticribra weissflogii*, which belongs to
300 the same family and is frequently misidentified due to taxonomic uncertainty surrounding these two
301 species. *Mytilus sp.* was the only target not identified to the species level (mostly due to presence of
302 other closely related species and hybrids with other congeneric species). The percentage of spiked
303 species recovered by the JAMP pipeline in mock communities 1-5 was 85, 90, 90, 80, and 90,
304 respectively (Supplementary Table 2). All 8 phyla were recovered in each mock community. Only two
305 of the 20 species (*Cordylophora caspia* and *Arenicola marina*) were not detected in any of the five
306 communities (Supplementary Table 5). For each mock community, the percentage of expected reads
307 and the percentage of observed reads per species are shown in Supplementary Table 2. The
308 correlation between the observed and expected percent of reads was found to be positive and
309 significant ($r(98) = 0.39, p < 0.001$).

310 *Environmental samples*

311 Evidence of inhibition was found in 22 out of 156 samples, including seven low volume water and 13
312 tow net samples (Supplementary Table 6). One cycle (C_t) delay from the IPC control was considered as
313 evidence of inhibition. Six of the samples failed in the amplification process and were discarded from
314 the final analyses (Supplementary Table 6); one DLV water, one HV water, and four sediment sample
315 replicates. Raw OTU assignments can be found in Supplementary table 7.

316 The number of both OTUs and NIS detected was significantly higher for tow net samples compared to
317 any other sampling method. Shapiro Wilk tests for both the number of OTUs ($W = 0.76852$ p-value =
318 $3.537e^{-08}$) and NIS ($W = 0.5946$ p-value = $1.67e^{-11}$) showed a non-normal distribution, therefore
319 Kruskal-Wallis and PERMANOVA nonparametric tests were performed. The PERMANOVA showed a
320 significant influence of the factor method (levels: SLV Filtration, DLV Filtration, Tow net, Sediment
321 core, HV sampler) in the number of NIS detected but not a significant influence of neither the location

322 (Dublin, Waterford, Galway, Carlingford) nor the number of NIS and the site type (Marina, Port or
 323 Open water) (Table 1).

324 **Table 1. The model output for a PERMANOVA based on Jaccard similarity index exploring the effect**
 325 **of eDNA sampling method, location, type of site and number of NIS on OTU dissimilarity. N_NIS is**
 326 **referring to the number of NIS found in the current study. DF: degrees of freedom; SumOfSqs: sum**
 327 **of squares.**

PERMANOVA	DF	SumOfSqs	R2	F	Pr(>F)
Method	4	1.2347	0.32407	6.5406	0.001***
Location	3	0.0980	0.02571	0.6919	0.600
Site type	2	0.1860	0.04883	1.9710	0.117
N_NIS	1	0.0732	0.01920	1.5501	0.204
Residual	47	2.2181	0.58219		
Total	57	3.8099	1.00000		
Signif.codes: 0 '****' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

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 329 Kruskal-Wallis tests were significant for both the Number of OTUs ($H = 31.004$, $df = 4$, $p\text{-value} = 3.056$
 330 e^{-06}) and NIS ($H = 13.621$ $df = 4$, $p\text{-value} = 0.008608$). Ad-hoc pairwise comparisons using Wilcoxon ra
 331 nk sum test showed tow net sampling as the significant level within method factor (Table 2). Tow net
 332 samples retrieved significantly more species than the other sampling methods. However, the highest
 333 number of NIS were detected in the DLV water filtration samples, but the differences were not signifi
 334 cant when compared with the rest of the methods (Table 2).

335 **Table 2. Ad hoc pairwise comparison to test 2-2 the methods on the number of OTUs (N_OTUS)**
 336 **and NIS (N_NIS) recovered. Matrix showing significance between levels of the factor “method”.**

N_OTUS	HV	DLV	Tow Net	Sediment	N_NIS	HV	DLV	Tow Net	Sediment
DLV	1				DLV	1			
Tow Net	0.00038	0.00032	-		Tow Net	0.050	0.039		
Sediment	1	0.49718	0.00036		Sediment	1	1	0.051	
SLV	1	1	0.00038	0.33622	SLV	1	1	0.051	1

337
 338 Different taxonomic composition was found depending on the method employed (Figure 1). The
 339 highest percentage of reads found by tow net samples and HV samplers belonged to diatoms,
 340 meanwhile the reads from filtered seawater were dominated by brown algae. Fungi was the most
 341 abundant taxa in sediment samples and arthropods and annelids were distributed along all sample
 342 methods, with tow nets recovering most of the reads belonging to arthropods taxa and sediment
 343 those with the highest matches with annelids. HV samples and tow nets recovered similar taxonomic
 344 compositions, with similar number of reads belonging to the same taxon, except for Chordata, which
 345 was better represented in tow net samples.

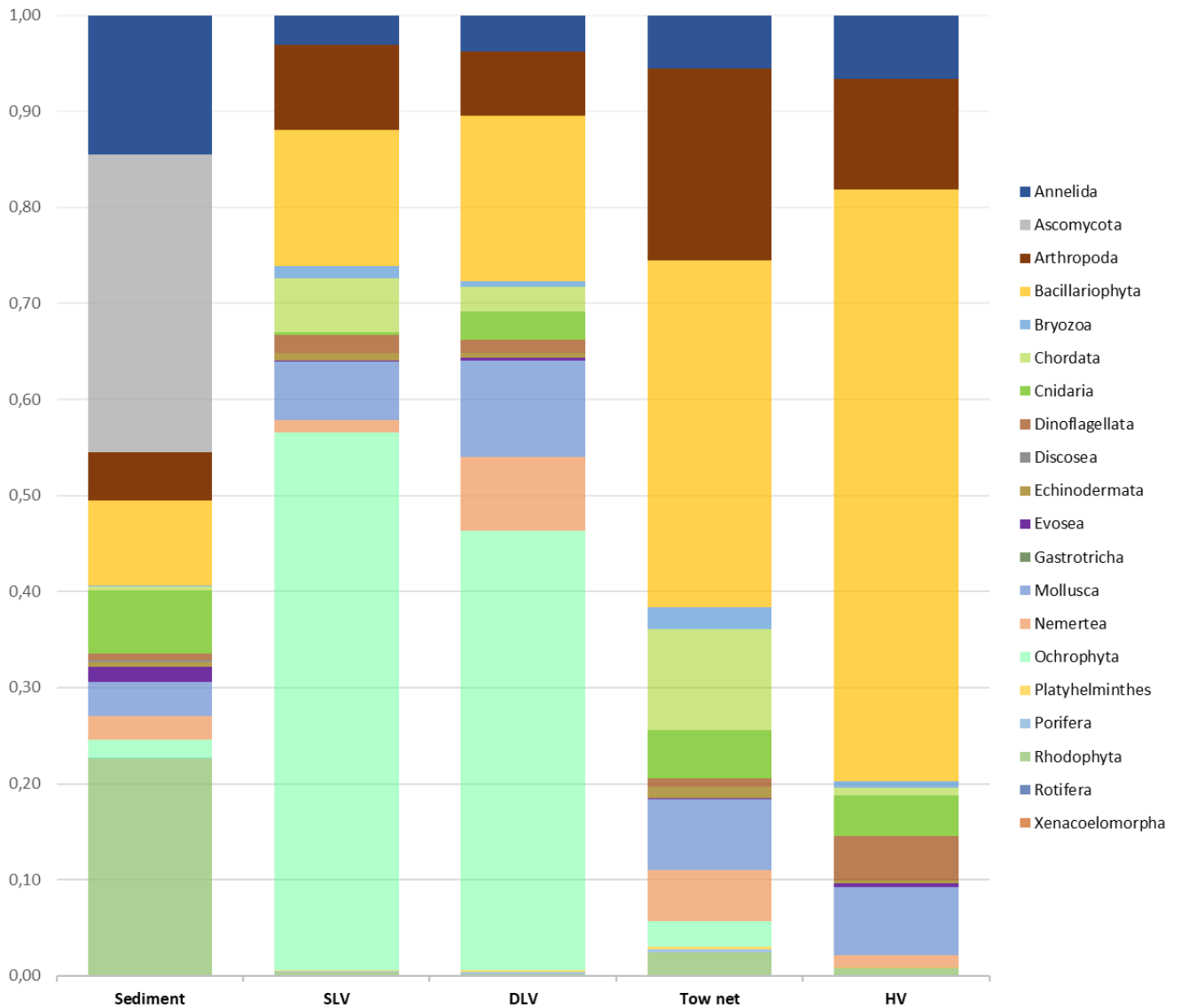
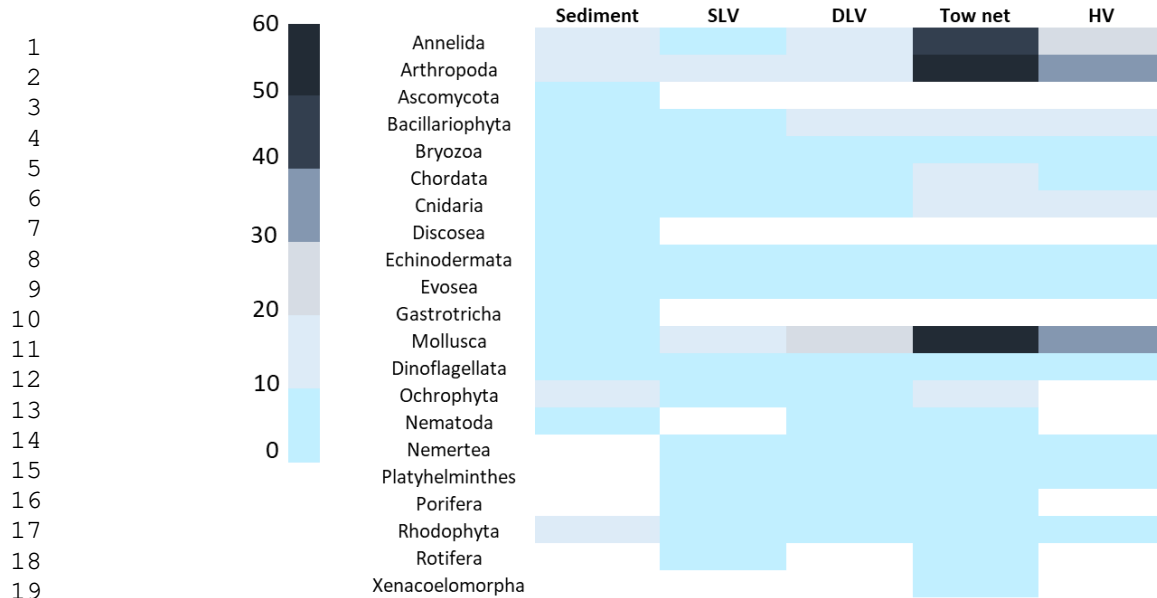


Figure 1. Proportion of reads belonging to different phyla per sampling method (SLV: Surface low-volume (3L); DLV: Deep low-volume (3L); HV: High-volume (50L)). Samples were pooled by field methodology across all sampling locations prior to analysis.

Tow net samples were richer in Annelida, Arthropoda, and Mollusca species than the rest of the sample types. Tow net samples were the most diverse with the highest number of species detected. Higher number of species was detected when using DLV samples than when using SLV, however, DLV were not able to detect Rotifera species (Figure 2). Rotifera are freshwater species so this is probably due to river Liffey influence in surface more than bottom samples in the site of detection.



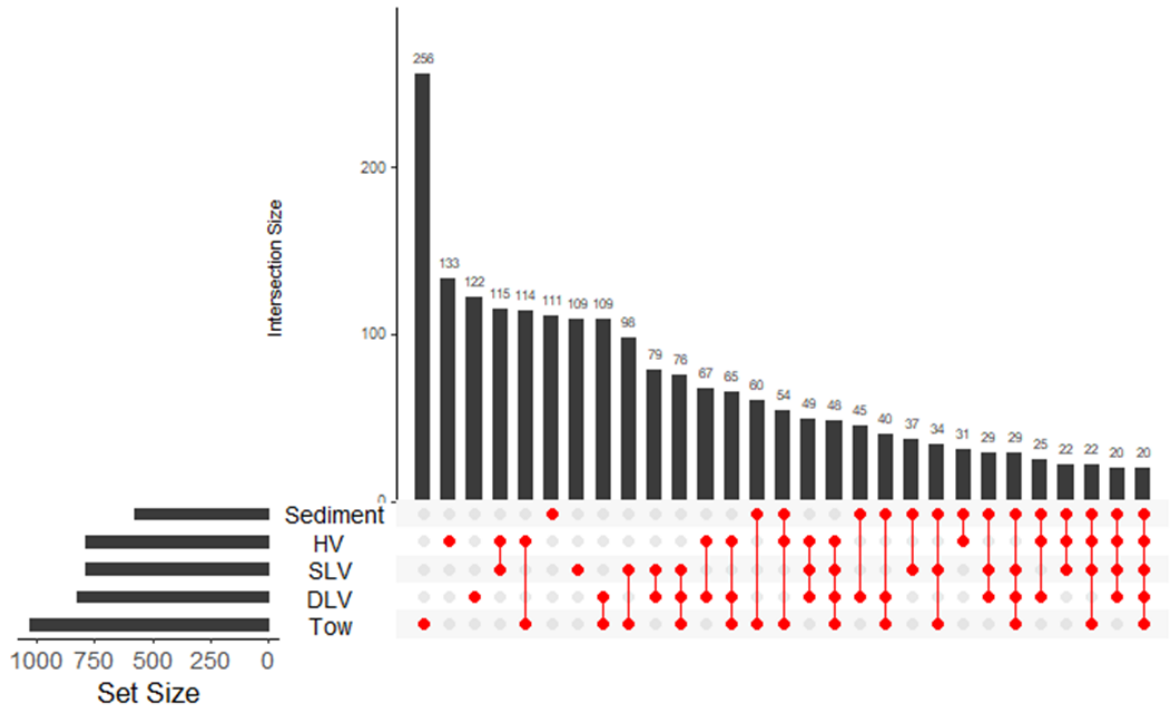
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Figure 2. Heatmap representing the number of Species (0-60) belonging to each taxon (from Annelida to Xenacoelomorpha) detected per sampling type (SLV: Surface low-volume (3L); DLV: Deep low-volume (3L); HV: High-volume (50L)).

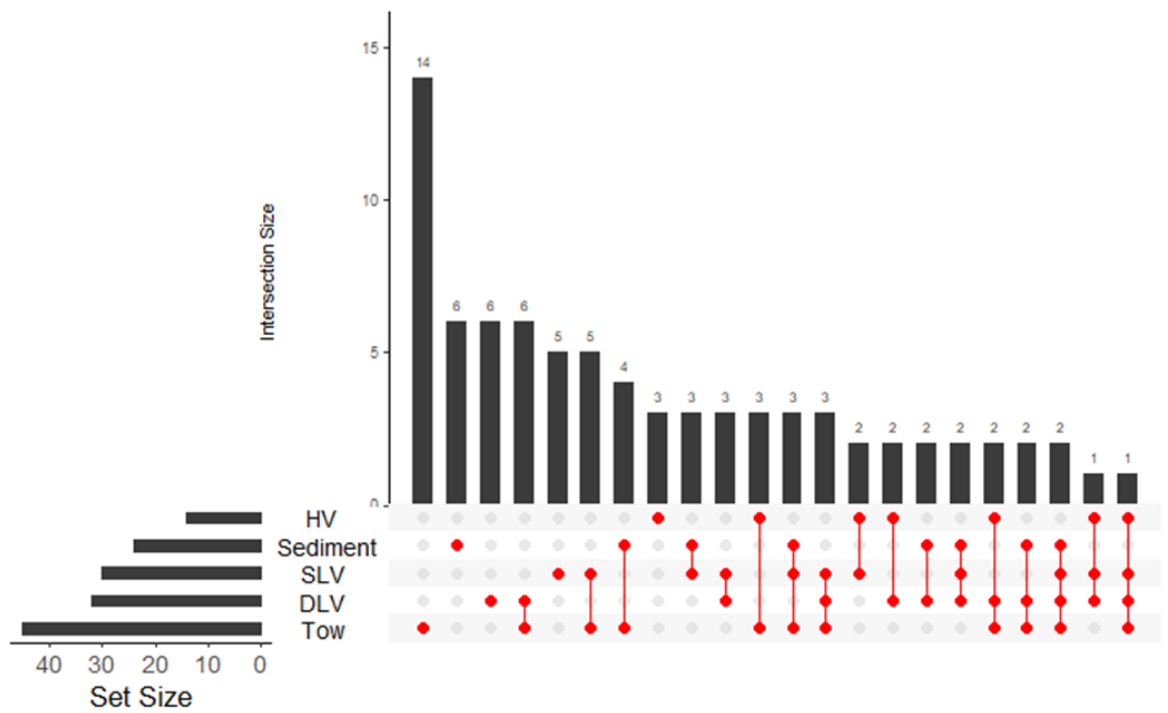
The Upset plot (Figure 3a) showed that 72 and 41 unique OTUs were detected from Tow nets and Sediment samples, respectively. Five and four in the case of water filters (Surface and Deep respectively), and 15 by HV samples (Figure 3a). Sediment samples contained the highest number of uniquely detected species belonging to Rhodophyta (14) and tow net samples belonging to Arthropoda (23), Annelida (12) and Mollusca (11). Upset plot from NIS data illustrates that four unique species were detected by tow nets and two by sediment samples (Figure 3b). The number of both NIS and OTUs found by the filter method was higher when collecting DLV samples than when collecting SLV seawater. HV samplers found the lowest number of NIS, followed by sediment. A total number of 16 NIS species were found using all sampling methods. Two were not detected using tow net samples (*Sargassum muticum* and *Watersipora subatra*), both uniquely detected when using sediment samples. Three NIS species were found using HV samplers (*Mya arenaria*, *Ostrea angasi* and *Ruditapes philippinarum*). *Acartia tonsa*, *Caprella mutica*, *Mytilicola intestinalis* and *Mytilicola orientalis* were detected only when using tow net samples. *Ostrea angasi*, *Ficopomatus enigmaticus* and *Botrylloides*

372 *violaceus* were found only in DLV seawater, not in SLV seawater. *Dasysiphonia japonica* and
 373 *Melanothamnus harveyi* were found in SLV seawater and not in DLV seawater samples.

a



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375 **Figure 3. Upset plot representing number of OTUs (a) and NIS (b) per method and how they overlap.**

376 **Set Size: total number of hits overlapping. Intersection size: number of OTUs (a) and NIS (b) in the**
377 **intersection. Red lines represent the methods that share the number of species specified on the bar**
378 **plot.**

379 Regarding location, the highest number of NIS was found in Carlingford, followed by Dublin. Waterford
380 was the location with less NIS detected (Figure 4) and the only location where *Acartia tonsa* was
381 detected. There are no previous records of *A. tonsa* in Ireland. *Bonnemaisonia hamifera*, *Ruditapes*
382 *philippinarum* and *Ostrea angasi* were only detected in Galway, while *Ficopomatus enigmaticus* was
383 only detected in Dublin. A significant difference in NIS richness was found between Carlingford and
384 Waterford (Kruskal-Wallis chi-squared = 13.251, df = 3, p-value = 0.004125; Pairwise comparisons
385 using Wilcoxon rank sum test P-value = 0.0032), which is in line with the large difference in detected
386 NIS.

387 Each NIS OTU assignment was confirmed using a BLASTn search against the entire NCBI nucleotide
388 collection (nt/nr) via the online NCBI portal (<https://blast.ncbi.nlm.nih.gov/Blast>, last accessed
389 07/12/2020). Reads assigned as *Ostrea angasi* could belong to *Ostrea edulis*. Reads assigned as
390 *Sargassum muticum*, can also be *Sargassum confusum* or *Sargassum nahozouense* so both
391 assignments should be considered to reach genus level.

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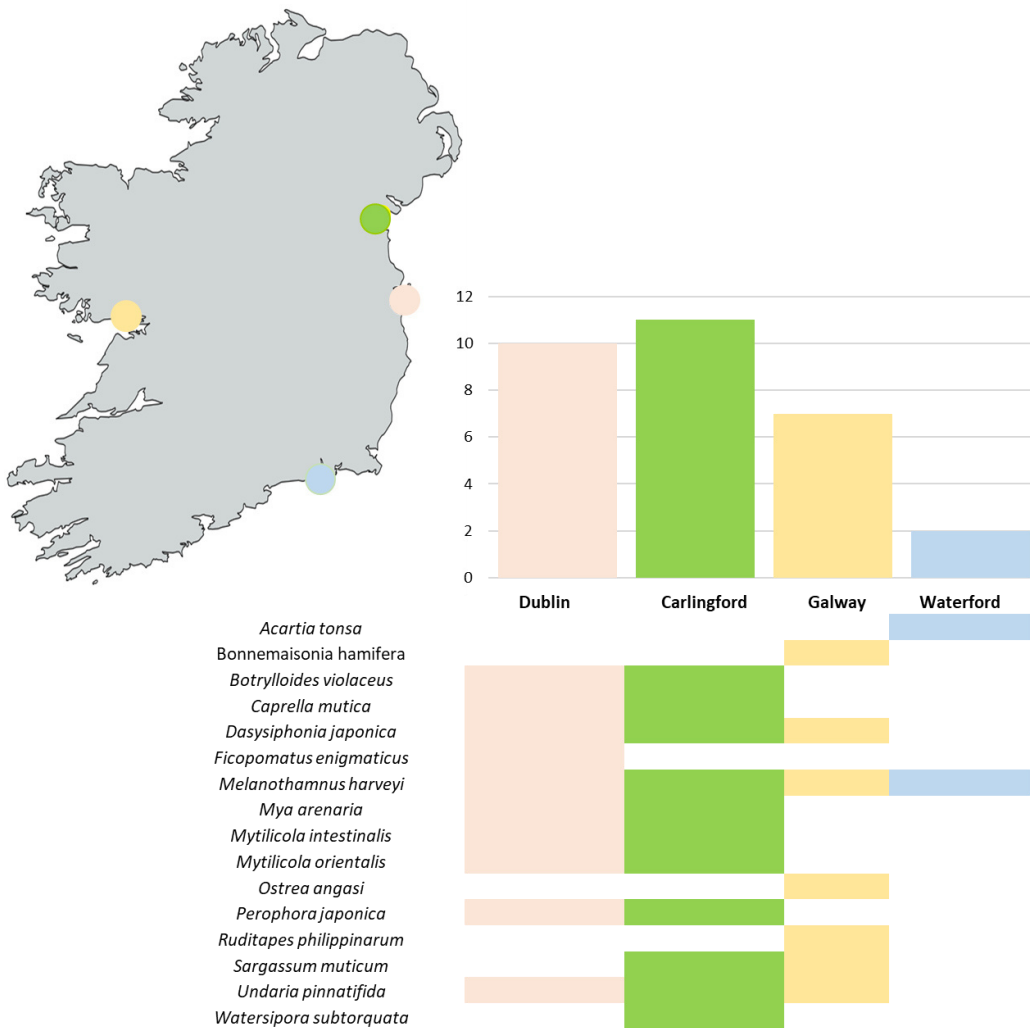


Figure 4. Each species detected per location and map showing where the locations are. Each species detection per location is shown in different colours depending and using species names.

The level of impact of the 16 NIS detected varies, *Bonnemaisonia hamifera*, *Botrylloides violaceus*, *Sargassum muticum* and *Undaria pinnatifida* are considered to have a high impact in Irish waters, meanwhile the rest of NIS have less impact or has not been well described yet (Table 3). There is one NIS not previously reported in Ireland, *Acartia tonsa*.

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Table 3. NIS found in the current study and the information regarding the Irish status, records on Global IS database, impacts from those with previously reported impacts and origin of the introduction.

Species name	Irish status	Origin	Known Impacts	High Impact	Global invasive species database
<i>Acartia tonsa</i>	Not present	Indo-Pacific. North-East Atlantic			NO
<i>Bonnemaisonia hamifera</i>	Established	Unclear		X	NO
<i>Botrylloides violaceus</i>	Established	North East Pacific			NO
<i>Caprella mutica</i>	Established	NW Pacific		X	NO
<i>Dasysiphonia japonica</i>	Established	NW Pacific			NO
<i>Ficopomatus enigmaticus</i>	Established	Australia	Significant change in ecological and sedimentary dynamics.		YES
<i>Melanothamnus harveyi</i>	Established	Tropics			NO
<i>Mya arenaria</i>	Established	NW Atlantic	Benthic-pelagic interaction, bioaccumulation, community dominance and habitat change.		YES
<i>Mytilicola intestinalis</i>	Established	Mediterranean			NO
<i>Mytilicola orientalis</i>	Reported	Sea of Japan			NO
<i>Ostrea angasi</i>	Reported				NO
<i>Perophora japonica</i>	Reported	NW Pacific			NO
<i>Ruditapes philippinarum</i>	Reported	W Tropical Pacific			NO
<i>Sargassum muticum</i>	Established	NW Pacific		X	NO
<i>Undaria pinnatifida</i>	Established	NW Pacific	Change the structure of the ecosystem. Depends on the location.	X	YES
<i>Watersipora subtorquata</i>	Reported	Unclear	Facilitates the spread of other invasives by providing a non-toxic surface for other fouling species to settle.		YES

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404 **Discussion**

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2 405 In this study, we tested a range of eDNA sampling approaches to be used in future monitoring and
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4 406 surveillance programs for NIS in Irish coastal waters. A wide range of both indigenous and non-
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6 407 indigenous taxa was successfully recovered across all four sampling techniques and across different
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9 408 sampling environments.

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12 409 The use of mock communities (mixtures of genomic DNA from known NIS) proved to be a crucial step
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14 410 to evaluate the success of the eDNA metabarcoding pipeline implemented in this study. The expected
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16 411 percentage of reads from those communities differed from the final values, but there was a positive
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18 412 significant correlation between them, thus we can conclude that the implemented pipeline is efficient.
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20 413 However, *Cordylophora caspia* and *Arenicola marina* were not detected in any of the five
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22 414 communities, which may have been due to primer competitiveness or selectivity as uneven amplification
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24 415 of universal primers is typical in metabarcoding experiments (Piñol et al., 2018). One way to deal with
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26 416 this limitation, is the use of several primer sets within the same genetic region (e.g. Lacoursière-
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28 417 Roussel et al., 2018a), but it would also increase the costs, so it is a matter of balance between costs
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30 418 and efficiency.

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37 419 Direct quantitative assessments based on the differences in proportions of reads are not always
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39 420 appropriate as shown in Supplementary Table 2, and in previously published research (Fernández et
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41 421 al., 2018; Zaiko et al., 2018). In addition, some of the sequences from the mock community were
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43 422 assigned against species not added to the mock community (Supplementary table 5), therefore a
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45 423 number of false positive detections should be considered as part of the pipeline and must be reduced
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47 424 or accounted for following the guidelines suggested by Ficetola, Taberlet, and Coissac (2016). Indeed,
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49 425 the genus level reassignments of two of the NIS annotated by the automated pipeline (*Ostrea angasi*
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51 426 and *Sargasum muticum*) underline the importance of manually checking lists of species identifications
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53 427 where DNA reference data is incomplete. As discussed in Darling et al. (2020) without strict shared
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1 428 quality assurance, protocol and reporting standards DNA-based biomonitoring should not be used
2 429 alone as evidence eliciting a management response.
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5 430 Another key technical consideration when working with eDNA is the possibility of PCR inhibition. We
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7 431 found tow nets to be the most affected sample type by inhibition, although similar numbers of reads
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9 432 were obtained in the rest of the samples (Supplementary table 6). Tow net samples were also the
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11 433 most affected by contamination, with nine blanks compromised, probably due to the highest amount
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13 434 of DNA present in this type of sample and/or suboptimal decontamination procedures. Limited
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15 435 information is available about the recommended proportion of controls in an eDNA workflow
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17 436 (Goldberg et al., 2016) and each study should determine how many negative controls to apply
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19 437 depending on the research question or purpose of the study. In the present study, we implemented a
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21 438 large number of negative controls (n = 47) to provide further insights on the tested protocols, however
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23 439 in the context of detecting rare or newly introduced NIS as part of future monitoring programs, we
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25 440 recommend the inclusion of field negative controls especially when sampling gear is reutilized despite
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27 441 decontamination.
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35 442 Notwithstanding above-mentioned disadvantages, tow net sampling has shown to be the most
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37 443 complete method to detect the largest number of species, finding the highest number of species and
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39 444 NIS (72 unique OTUs and four unique NIS). It did not detect two of the NIS (*Sargassum muticum* and
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41 445 *Watersipora subatra*), both only detected in sediment samples, which could be due to the habitat of
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43 446 the species at the sampling timepoint, as both are normally fixed to solid substrates. Therefore, we
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45 447 have established in a metabarcoding context that low volume water filtering may not be the preferred
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47 448 eDNA sampling method as it has been previously suggested (Schabacker et al., 2020).
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52 449 Some of the results showed here are not aligned with previously published data where tow nets were
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54 450 employed (Djurhuus et al., 2018; Sepulveda et al., 2019). The main difference is the mesh size of the
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56 451 tow net, while the smallest mesh size was used by Djurhuus et al. (2018) with 64 μm , here we
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58 452 employed 40 μm mesh. This explains the increased diversity recovered compared to other sampling
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1 453 techniques. Moreover, none of those studies tested for inhibition, which could affect the biodiversity
2 454 recovered. Koziol et al. (2019) found that sediments recovered the largest number of OTUs, but they
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4 455 included an additional inhibition removal step for sediment samples that could have contributed to
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7 456 the increased efficiency.

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10 457 As expected, for being high risk locations due to the highest concentration of NIS introduction vectors
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12 458 (Tidbury et al., 2016), more NIS were detected in Dublin and Carlingford (N= 10 and N= 11 respectively;
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15 459 Figure 6), and fewer NIS were detected in Galway (N=7) and the Waterford estuary (N=2)(Figure 6).
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17 460 The NIS species detected in Waterford were *Acartia tonsa* that was only detected in this location and
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19 461 *Melanothammus harveyi* also detected in the other three locations. The presence of *Acartia tonsa*
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22 462 Dana (1849), a planktonic crustacean (calanoid copepod), in Irish waters is not surprising, however to
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24 463 the best of our knowledge this species has never been officially reported in Ireland. While eDNA does
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27 464 not confirm the presence of a biologically active organism, in the context of a surveillance program, it
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29 465 can act as sentinel method to inform a more targeted follow-up survey using both molecular methods
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32 466 (Moseid et al., 2021) and non-molecular methods, whereby the actual NIS is captured and
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34 467 taxonomically identified.

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37 468 These findings further highlight the utility of including eDNA approaches in monitoring and
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40 469 surveillance programs of NIS where early detection is important (Ammon et al., 2018; Brown et al.,
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42 470 2016). The combination of different sampling types results in a greater proportion of total taxa
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44 471 detected. However, it is more costly and can be time consuming (Evans et al., 2017; Hayes et al., 2005).
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47 472 To reduce costs (i.e. limit number of total samples by lowering number of sampling types) or to
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49 473 improve probability of detection (i.e. exchange number of sampling types for increased replicates),
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52 474 we recommend choosing the sampling technique based on the targeted taxa group (taking the target's
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54 475 life history traits into account) before implementing surveillance programs. Sampling type must be
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56 476 considered as the substrate can dramatically influence the number of species and which taxa are
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59 477 detected as it has been previously described in Koziol et al.(2018). In the present study, fish species
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478 were not detected when collecting sediment and HV samples, as opposed to Holman et al. (2019)
479 where fish species were found in both water and sediments, however, they also used 18S marker what
480 could influence this difference. In comparison, Fungi, Discosea and Gastrotricha species were only
481 detected in sediment samples. If the target group is brown algae, then, water samples are the
482 recommended approach; meanwhile, if targeting platyhelminths, either tow net or HV samples would
483 be better choices. Furthermore, other factors such as abundance, behaviour, niches occupancy and
484 life cycle of target NIS can affect probability of detection, thus eDNA sampling approaches should be
485 adapted accordingly.

486 Regarding the diversity found in each sampling technique, tow net and HV samples detected more
487 arthropods, annelids, and mollusks; thus, we expect these sampling methods to be the most efficient
488 at targeting those groups. If sediment samples are excluded, for being the most dissimilar *a priori*,
489 when tow net samples are compared with water (SLV, DLV, and HV), it has been the only technique
490 able to detect all the taxonomic groups, as Nematoda was only found by DLV samples and Rotifera by
491 SLV samples, meanwhile, tow net samples have detected both (Figure 2).

492 Based on the results obtained in the current study, a combination of techniques is recommended to
493 maximize the number of species detected. However, if only a single method can be deployed, our
494 results showed that among those tested, tow net sampling are recommended. Mollusk species are
495 among the most abundant NIS (Ardura, 2019; Clusa et al., 2017; Molnar et al., 2008b), and in the
496 present study tow nets detected the highest diversity of mollusks (followed by HV, DLV, SLV and finally
497 sediment samples), as expected considering the larval state of mollusks is found in water opposed to
498 sediments, and is therefore the most efficient method for the detection of these species.

499 Findings from the present study allowed the testing, validation and assessment of a range of eDNA
500 sampling protocols and approaches that will be at disposal of future marine NIS monitoring and
501 surveillance programs in Irish waters and adjacent areas.

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