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The use of environmental DNA metabarcoding and quantitative PCR for molecular detection of marine invasive non-native species associated with artificial structures.

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Abstract

Artificial coastal structures associated with coastal defences, energy generation, ports, marinas and other developments, are known to support lower levels of biodiversity than natural coastal environments and tend to be hotspots of invasive non-native species (INNS). In the present study, we attempted to detect INNS through both quantitative (q)PCR and metabarcoding of environmental (e)DNA from seawater samples. A mitochondrial COI based species-specific qPCR assay was developed and deployed to detect *Didemnum vexillum*, a colonial tunicate that has successfully become established at coastal sites across Europe. Our targeted qPCR assay was able to detect *D. vexillum* in eDNA seawater samples from all sampled sites where it is currently found in Ireland and Wales. Through metabarcoding of the same eDNA samples, we detected an established INNS at all sites but not *D. vexillum* even in locations were it is present. We conclude that our qPCR approach is effective for sensitive and targeted screening for specific INNS at coastal sites including those with artificial structures, and while metabarcoding is a less sensitive approach it is a valuable tool to detect a broad taxonomic range of native and non-native species.

Keywords: *Didemnum vexillum*; genetic markers; high throughput sequencing; INNS; Irish Sea; qPCR

Declarations

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Introduction:

Artificial structures in the coastal zone are essential to facilitate transport, recreation, aquaculture, renewable energy and defence against storms and erosion. They can include fixed structures such as sea walls and rock armour breakwaters, removable structures on the seabed such as aquaculture trestles and floating structures such as pontoons and buoys (Airoldi and Beck 2007; Bulleri and Chapman 2010; Kittinger and Ayers 2010; Firth et al. 2013). Artificial structures can support communities of marine organisms, but their relatively smooth surfaces tend to provide less habitat heterogeneity than natural rocky shores (Firth et al. 2016). There is a paucity of the natural crevices and pools that would normally facilitate water retention (Firth et al. 2013), as well as a lack of variable textures and overhangs to act as refugia and support diverse species assemblages (Connell, 1972; Strain et al. 2018; Evans et al. 2019, Evans et al. 2021). For these reasons, there are major differences in the composition of biological communities associated with coastal environments containing artificial structures and those that occur on natural rocky shores. For example, communities associated with artificial structures have been shown to exhibit lower biodiversity (Chapman & Bulleri, 2003; Bulleri & Chapman, 2004; Garcia et al. 2007; Vaselli et al. 2008; Pister, 2009) and support a higher proportion of invasive non-native species (INNS) (Airoldi & Bulleri, 2011; Firth et al. 2011; Mineur et al. 2012). The prevalence of the latter may in part be due to the urbanisation of estuarine habitats, with installation of artificial structures creating hard substrates where none have previously existed (Ruiz et al. 1997; Bacchiocchi & Airoldi 2003). These installations are often associated with shipping and aquaculture, and represent entry points for invasion, enhancing the spread and establishment of INNS at these locations (Glasby et al. 2007).

In recent years, research efforts have been focused on increasing the heterogeneity and water retention of artificial structures through ecologically sensitive engineering in coastal areas

(Chapman & Blockley, 2009; Browne & Chapman, 2011; Firth et al. 2014; Evans et al. 2015). Trials have indicated that measures such as the installation of artificial concrete rock pools (Hall et al. 2019), and the drilling of pits into existing structures (Evans et al. 2015) can provide opportunities for colonisation by a wide variety of species. However, the challenge remains to implement effective methods to assess and identify the species that are found at coastal locations containing artificial structures, and to detect the presence of INNS that may also potentially displace native biodiversity that exists in these regions.

Environmental DNA (eDNA) is a survey methodology that relies on the detection of taxa from extracellular and intracellular material that is deposited into the environment. Following isolation of this material from the environmental sample (such as water, air or soil; Taberlet et al. 2012) it can be interrogated in different ways. Targeted species detection can be undertaken through quantitative (q)PCR using specifically designed primers and probes (e.g. Ficetola et al. 2008; Jerde et al. 2011; Gustavson et al. 2015; Gargan et al. 2017), whereas a more general approach focusing on detection of multiple species is achieved using High Throughput Sequencing (HTS) and metabarcoding (e.g. Thomsen et al. 2012; Hänfling et al. 2016; Holman et al. 2019).

Due to the non-invasive genetic methods employed by eDNA studies, such approaches are increasingly used for invasive or cryptic species detection in marine ecosystems (Zaiko et al. 2015; Borrell et al. 2018; Holman et al. 2019). This is especially true where such species may exist in low abundance and may be difficult to detect using conventional survey methods (e.g. visual observation, netting or trawling etc.) or where conventional surveys are logistically complex and resource intensive. Taking water samples for eDNA surveys is more staightforward compared to expensive and complicated dive/ROV/camera sledge surveys which require highly qualified personal and dedicated vessels.

Early detection and intervention is particularly important for INNS such as the colonial ascidian *Didemnum vexillum* (Sambrook et al. 2014). This species is associated with artificial structures in ports and marinas, where it can rapidly spread and foul a wide variety of surfaces including coastal structures, aquaculture facilities and the hulls of ships (Bullard et al. 2007). Invasion by *D. vexillum* can lead to both ecological and economic impacts. Competition with native species can cause changes in habitat complexity and ecosystem function (Cordell et al. 2013), and rapid growth in aquaculture facilities and marinas can be problematic. Although it is native to the coastal waters of Japan (Stefaniak et al. 2012), *D. vexillum* has been spreading worldwide in recent decades, becoming successfully established in New Zealand, the east and west coasts of the United States, Canada, throughout the Mediterranean and northern Europe (Lambert 2009; Stefaniak et al. 2009; Tagliapietra et al. 2012; Vercaemer et al. 2015; Ordóňez et al. 2015; Fletcher et al. 2018). It was first identified in marinas in Ireland in 2006 (Minchin & Sides, 2006) and Wales in 2008 (Griffith et al. 2009), as well as other UK locations such as the southern English coast (Bishop et al. 2015) and Scotland (Beveridge et al. 2011). Once established, *D. vexillum* is very difficult to eradicate even in cases of relatively small, localised infestations as evidenced by two failed eradication attempts at Holyhead Marina, North Wales (Sambrook et al. 2014).

We assessed the utility of different eDNA approaches to determine the presence of INNS at natural and artificial coastal sites using *D. vexillum* as a model species. Simpson et al. (2017) previously developed and tested a qPCR assay for detection of this species in Australian waters. However, to date there have been no published studies implementing qPCR analysis of eDNA for the detection of the more genetically diverse populations of *D. vexillum* found in European waters (Graham et al. 2015). The effectiveness of metabarcoding analysis of eDNA samples as a monitoring tool for detecting established and newly introduced INNS species has been demonstrated at marinas on the English and Welsh coastlines (Holman et al. 2019).

153 Here we compare the effectiveness of both the general approach (metabarcoding) and specific
154 targeted approach (qPCR) to detect *D. vexillum*. We also make a general assessment of the
155 use of metabarcoding in detecting INNS by screening the resulting sequences against a list of
156 other regionally targeted INNS.

157

158 **Materials and Methods:**

159 *Site selection and water sampling:*

160 A total of six sites were selected for this study, comprising three sites on the east coast of
161 Ireland and three sites in North Wales (Figure 1). These sites represent three different study
162 site types. The first two types are sites that contain permanent large intertidal and floating
163 artificial structures such as floating pontoons, pilings and rock armour, either set in estuarine
164 conditions in an enclosed bay subject to high flushing and water retention during tidal cycles
165 (hereafter called "closed artificial" for simplicity) or set in fully marine coastal conditions
166 ("open artificial"). We also included a more natural coastal site type, where there are few
167 artificial structures present in the sampling area ("natural"). These sites were used as a
168 control for assessing the presence of INNS.

169

170 Sampling was carried out between late September and early October 2018. Water samples
171 were collected from three locations within each site; close to the coastline or marina, mid-
172 channel, and in the outer reaches of each site (see Table S1 for sampling locations). All water
173 samples were collected during ebb tide, to capture the broadest possible representation of
174 eDNA from our sample locations and minimise offshore influence. Each water sample
175 consisted of 2 L of seawater and samples were collected in triplicate (total n=9 samples for
176 each site). At Holyhead Marina where *D. vexillum* is known to be, an additional set of

177 samples was taken from within 1, of the pontoon sides (total *n*=12 samples for this sample
178 location). At all sites, water samples were taken from the side of a boat using gloved hands
179 and single-use plastic water bottles which had never previously contained seawater. Each
180 sample site also included a field control sample containing 2 L of shop-bought water, which
181 was opened momentarily during field work, stored and processed alongside eDNA samples
182 and used to test for contamination in the field. After sampling each site, water samples were
183 stored at 4^{o}C and processed in the lab within 24 hours of collection. Samples were vacuum
184 filtered through sterile Whatman 47 mm diameter, 0.45 µm pore size nylon filters. A
185 laboratory blank sample containing 1 L of ddH2O was filtered and processed along with the
186 eDNA samples to detect potential contamination during the filtering process. Filters were
187 stored in foil and frozen at -20^{o}C until DNA extraction.

188 In order to ensure the efficacy of our novel qPCR assay, an additional set of 2 L water
189 samples was taken from Malahide Marina (n=3) directly beside a colony of *D. vexillum* in
190 September 2019. This set of samples (including field, filtration and extraction control
191 samples), was processed in the same way as all other eDNA samples.

192

193 *Extraction of eDNA:*

194 DNA was extracted from half filters using QIAshredder (Qiagen; to homogenise the DNA
195 found on the filter) followed by the DNeasy Blood & Tissue Kit (Qiagen) according to the
196 manufacturer's instructions and eluted in a final volume of 50 µl AE buffer (supplied with
197 kit). eDNA concentrations were determined using spectrophotometry (Biodrop µLite,
198 Biodrop; see Table S1). To decrease the risk of contamination, sterile filtering equipment,
199 multiple-glove changes, and separate dedicated lab spaces for eDNA extraction, pre-PCR and
200 post-PCR processing were employed. Samples from each site (including their respective field

and filtration blank samples) were processed as separate sets of samples in the lab, and each set of samples included an extraction blank to test for contamination during the extraction process.

qPCR assay development:

A qPCR analysis of our water samples was implemented to specifically screen for *D. vexillum.* This species was observed (through conventional survey methods) to have been present at three of the study sites for over a decade (i.e. Malahide Marina and Carlingford Lough (Minchin 2007) and Holyhead Marina (Griffith *et al.* 2009)) and was observed to be still present at the study sites within 18 months of water sampling (Ireland: Personal observations by authors and Malahide marina staff; Wales: Holt (2019).

Sequences of the mitochondrial *COI* region for European *D. vexillum* individuals, publicly available on GenBank, were visualised in Geneious (R8, Biomatters Limited). A consensus sequence was generated for the target species incorporating any intraspecific variability for the *COI* region (see Table S2 for accession numbers of unique sequences used to generate the consensus sequence).

A qPCR assay for *D. vexillum* detection has previously been developed and tested in Australia (Simpson *et al.* 2017). The primers and probes from this assay were checked against the sequences of *D. vexillum* occurring in European waters. Due to the intraspecific diversity occurring in *D. vexillum*, there was a mismatch between some individuals of European *D. vexillum* and the previously published assay, which necessitated designing a new assay for use in our study. Amending the existing assay would have entailed incorporating degeneracy in the probe that may have compromised assay specificity and/or efficiency (see Figure S1). Therefore, it was necessary to design new primers and probes

based on sequences from those individuals occurring throughout Europe. The novel assay designed for this study comprises forward (*Dvex-F1* 5'-TGA GCT GCT ATA GTT MGA GCT AGA TTT AGT-3') and reverse (*Dvex-R1* 5'- TTC AAA CGR GGA AAA GCT ATA TC-3') primers, and a minor-groove binding (MGB) probe incorporating a 5' reporter dye and a 3' nonfluorescent quencher (*Dvex-PR* 5'-ATA ATT TTG TTA TCA CGG CTC AT-3'). The assay was designed using Primer Express (V3.0, Life Technologies) and targets a 221 bp region of the mitochondrial *COI* gene. The specificity of the generated primers and probe were checked against the NCBI (http://www.ncbi.nlm.nih.gov/) nucleotide database. An overview of primers used in this study is available in Table S5.

qPCR analysis:

Assay optimisation was carried out using tissue-extracted DNA from *D. vexillum* colonies collected in Ireland and Wales. The qPCR consisted of a 30 µl reaction volume containing 15 µl of TaqMan® Environmental Master Mix 2.0 (Life Technologies, Applied Biosystems, Foster City, CA), 3 µl of each primer (final concentration of 2 µM), probe (final concentration of 2 µM), 6 µl DNA template and ddH2O. The PCR program consisted of 95ºC for 10 min, followed by 45 cycles of 95ºC for 15 s and 55ºC for 90 s. All qPCR reactions were carried out using the QuantStudio™ 7 Flex Real-Time PCR System (Life Technologies, Applied Biosystems, Foster City, CA). Once assay optimisation was complete, eDNA samples were run in triplicate in the qPCR assay, along with technical blanks and a 7-point serial dilution (10:1) of tissue-extracted DNA from *D. vexillum* colony samples. Concentrations for the serial dilution ranged from 180 ng to 180 x 10^{-6} ng (or 0.18 pg) of DNA in each reaction based on spectrophotometer quantification (Biodrop). The standard curve for our *D. vexillum* assay ($y = -3.413x+25.904$, $r^2 = 0.998$, efficiency = 96%) was

generated using 6 µl of template in a total reaction volume of 30 µl. A positive detection was established to be any sample that amplified in at least two out of three technical replicates and was further verified by Sanger sequencing of the PCR product (the highest Ct 40.8) . Where possible, positive detections were quantified based on the standard curve. However, for the scope of the current study, presence versus absence of the target species was deemed sufficient to determine if the species was found at our study sites.

Sanger sequencing of product from positive qPCR reactions was carried out to verify detections. PCR product from one replicate of each of the positive samples was purified (using ExoSAP-IT™, according to manufacturer's guidelines). Sanger sequencing took place at a commercial facility (Macrogen, Europe), in both forward and reverse directions. The resulting sequences were aligned against a reference consensus *COI* sequence in Geneious and checked by eye for any ambiguous or erroneous base calls. Sequences were then checked against the NCBI database.

Metabarcoding tag design and primer selection:

Unique identification tags were generated for each sample using the program OligoTag (Coissac 2012), specifying a length of 8bp, a minimum hamming distance of 3 and containing no homopolymers. These tags were designed in order to facilitate downstream demultiplexing of each sample that was sequenced in the final library. Primers were ordered with the tags incorporated on the 5' end (on both forward and reverse primers). The *COI* gene is widely used as the barcoding marker of choice for animals (Hebert et al. 2003), and one of the main advantages of using *COI* for eDNA metabarcoding is the existence of growing reference databases and initiatives for this marker (e.g. The Consortium for the Barcode of Life (CBOL, http://www.barcodinglife.org)). The primers selected for his study included

degenerate primers to enable amplification of a wide range of marine organisms from our eDNA samples. The primers chosen were jgHCO2198 and jgLCO1490 (Geller et al. 2013) and LoboF1 and LoboR1 (Lobo et al. 2013). Primer sequences from both primer sets were mapped to the existing full mitochondrial genomes on GenBank (accession numbers: KM259616, KM259617 and NC026107) using Geneious (default settings). Primer sequences for primers used in this study are available in Table S5.

PCR and sequencing preparation:

Each sample was amplified using a designated unique set of tagged primers. PCR was carried out in triplicate for each sample and these PCR reactions consisted of 12.5 µl of Qiagen Multiplex PCR Master Mix (Qiagen), 5 µl of 10x primer mix (Integrated DNA Technologies, Inc.) and 2 µl of template DNA in a total volume of 25 µl. The 10x primer mix was created for each individually tagged primer set and consisted of equimolar concentrations (0.2 µM) of primers, according to the manufacturer's instructions. PCR was carried out under the following cycling conditions: initial denaturation at 95^{o}C for 15 min followed by 35 cycles of 94^{o}C for 30 s, annealing at 60^{o}C for 90 s and extension at 72^{o}C for 90 s. A final extension step was carried out at 72^{o}C for 10 min. PCR products were visualised on a 1% agarose gel stained with SYBR® Safe (Life Technologies) and a 1kb ladder (Solis BioDyne).

PCR replicates were pooled prior to purification and quantification. PCR product was cleaned using ExoSAP-IT™ (ThermoFisher Scientific), according to manufacturer's instructions. The concentrations of PCR products were individually measured by fluorometry (Qubit, ThermoFisher Scientific), and then each sample was added to the final library in equimolar concentration.

For this study, a total of 57 eDNA samples were included in the metabarcoding effort. Control samples originating from field, filtration and extraction blanks were also subjected to PCR, pooled and sequenced along with NTCs from PCR multiplexing. All samples were combined in a single library and sequenced using Illumina MiSeq V3 2 x 300bp chemistry. Final library preparation and sequencing was carried out by a commercial facility (Fasteris, Switzerland) using the MetaFast protocol (https://www.fasteris.com/dna/?q=content/metafast-protocol-amplicon-metagenomic-analysis).

Bioinformatics and statistical analysis of metabarcoding data:

The sequenced library was demultiplexed using cutadapt (version 1.7.1; Martin 2011), with a maximum of one error allowed per tag (each tag differed by at least three nucleotides). The quality of the demultiplexed samples was determined using FastQC (version 0.11.9; available at http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), at which point it was determined that the majority of reverse reads were of poor quality. Therefore, only the forward reads from our sequencing effort were retained for downstream analysis. The majority of downstream bioinformatics was carried out using Qiime2 (version 2019.10.0; Boylen *et al.* 2019). The forward reads from demultiplexed samples were trimmed, denoised, and dereplicated into unique Amplicon Sequence Variants (ASV's, constituting 100% identity between sequences) using DADA2 (Callahan *et al.* 2016). In this step, primers were trimmed from the 5' end of the reads. In addition, only those reads with a maximum of two expected errors were retained. Sequences identified as chimeric and sequences represented by a single read (singletons) were also discarded at this stage of the analysis. The dereplicated representative sequences (each constituting an ASV) and resulting ASV table were used for

taxonomic assignment. At this stage, we employed a conservative approach and any ASV that was detected in our control samples were also removed from the eDNA samples.

For taxonomic assignment, replicate eDNA samples were combined into site-level samples, resulting in a total of six samples representing each study site. Taxonomy was assigned to sequences (qiime feature-classifier) using Qiime2. We implemented the 'classify-consensus-blast' method, which performs BLAST+ local alignment between query and reference reads. Taxonomic assignment was accepted only where there was percent identity between query and reference reads of >97%, query coverage of >90%, and choosing the consensus among the top ten hits in our reference database. In this study, we used the MIDORI_UNIQ reference database for *COI* (Machida *et al.* 2017) as this contains a curated taxonomy and reference sequences from GenBank. Species-level taxonomic assignments were compared with species lists from previously published rapid assessment survey (RAS) data from our artificial sites in Ireland and Wales (Minchin 2007; Wood et al. 2015), where fouling assemblages on harbour structures were visually inspected for targeted INNS. See Table S3 for the list of INNS reported at these sites from RAS (Minchin, 2007) and NBN surveys (i.e. NBN Atlas, https://nbnatlas.org/). We also compared taxonomic assignments to online databases, World Register of Marine Species (WoRMS; http://www.marinespecies.org/) and AlgaeBase (https://www.algaebase.org/) to determine the establishment status of each species in Ireland and the UK.

Results:

qPCR detection of Didemnum vexillum:

A total of 60 eDNA samples were analysed using the qPCR assay (n=57 from samples taken across six sample locations in 2018 and n=3 taken from directly beside a *D. vexillum* colony

343 at Malahide in 2019). Of these 60 samples, 14 were positive for *D. vexillum* detection using
344 the qPCR assay. This included positive detection in samples taken in 2018 at Malahide (six
345 detections/nine samples), Carlingford Lough (four detections/nine samples) and Holyhead
346 (one detection/12 samples) (Table1). In the case of Holyhead samples, positive qPCR
347 detections were found in a single sample taken from within 1m of the pontoons. All samples
348 that were taken from directly beside *D. vexillum* colonies at Malahide in 2019 were positive
349 for detection of the target species (three detections/three samples). No detections were found
350 at Port Oriel, Conwy and Porth Dinllaen. None of the negative control samples (field,
351 laboratory filter, extraction or technical) resulted in any detectable amplification.

352

353 **Table 1:** Details of sample sites that were included in this study, including site type (where
354 open artificial and open natural describe coastal sites with and without artificial structures,
355 respectively, and closed sites are estuarine) and whether *Didemnum vexillum* was detected
356 using qPCR or metabarcoding of eDNA samples. See Figure 1 for location of sample sites.

Site name	Site type	Country	No. eDNA samples	Known *D. vexillum* presence	*D. vexillum* detection with qPCR	*D. vexillum* detection with metabarcoding
Port Oriel	Open natural	Ireland	9	No	No	No
Porth Dinllaen	Open natural	Wales	9	No	No	No
Carlingford Lough	Open artificial	Ireland	9	Yes	Yes	No
Holyhead Marina	Open artificial	Wales	12	Yes	Yes	No
Malahide Marina	Closed artificial	Ireland	9	Yes	Yes	No
Conwy	Closed artificial	Wales	9	No	No	No

357

At Malahide, where water samples were taken from directly beside *D. vexillum* colonies in 2019, all samples fell within our dynamic range and the average concentration of target DNA in the analysed samples determined by qPCR was 36.7 pg (SD 23.3). For those samples taken around the wider Malahide site in 2018, the average concentration determined by qPCR was 2.04 pg (SD 0.86). A single eDNA sample from Carlingford Lough contained an average concentration of 0.31 pg of target DNA. We were unable to accurately quantify the amount of *D. vexillum* DNA that was found in all positive samples as amplification fell outside of our dynamic range. A sample from Holyhead displayed late amplification at an average *Cq*=42 and was outside of the dynamic range for quantification but was considered indicative of species presence in that location. Sanger sequencing of PCR product from positive detections showed ≥97% identity to *D. vexillum* when compared against the NCBI database, including the sample from Holyhead that showed late amplification according to our qPCR analysis.

Metabarcoding results:

Between one and four mismatches were detected when mapping the COI primers used for metabarcoding to existing full mitochondrial genomes for *D. vexillum* from GenBank. (Figure S2). Our HTS metabarcoding effort resulted in a total of 24,295,984 raw reads. Following demultiplexing using cutadapt, 10,897,666 reads remained. After trimming, filtering, denoising, and chimera screening of forward reads using DADA2, 4,380,642 clean reads (18.03% of raw reads) of 230bp in length were retained for further analysis. Of these reads, 4,069,704 (~93%) reads were assigned to the 57 eDNA samples, with an average number of 71,398 (SD 35,850) reads per sample. The remaining number of reads were assigned across the control samples (consisting of PCR NTC, field, filter and extraction blank samples; total number of reads 304,689 or ~7% of total clean reads). Removal of all reads

belonging to ASV's that were found in the control samples from the eDNA samples, resulted in a total of 2,077,075 reads remaining for downstream analysis and taxonomic assignment (average of 36,439 (SD 20,395) reads per sample). Following removal of those ASV's found in control samples, the minimum number of reads per sample was 4,418.

Pooling of samples from each location into the six site-level samples resulted in an average of 346,179 (SD 57,351) reads per site (Conwy 435,451, Holyhead 350,107, Porth Dinllaen 356,223, Malahide 262367, Carlingford 360,411 and Port Oriel 312,516, respectively) and an average of 5,977 ASV's detected at each site (total 12,703 unique ASV's across all samples), to which we attempted to assign taxonomy. The highest number of ASV's were detected at Conwy in Wales (8,555 ASV's), with the lowest number found at Malahide in Ireland (3,864 ASV's). Taxonomy was successfully assigned to 131 ASV's across all samples, with 47 unique species identified. This corresponds to ~1% of all ASV's being assigned to species-level taxonomy from our dataset. Of these taxonomic assignments, all were deemed to be marine-dwelling organisms (with taxonomic assignment checked against online databases outlined in the materials and methods). The two closed artificial sites showed the lowest number of species per sequence (Conwy and Malahide 2.30 and 3.43 species per 100k sequences, respectively), while the open artificial sites showed intermediate numbers (Holyhead and Carlingford 4.28 and 3.61 species per 100k sequences, respectively) and the natural sites the highest (Port Oriel and Porth Dinllaen 8.96 and 4.77 species per 100k sequences, respectively).

A total of 14 distinct phyla were identified across sample sites (Figure 2) with the greatest number of phyla identified at Port Oriel and Porth Dinllaen. The most diverse phylum (in terms of number of ASV's detected) was the phylum Mollusca, comprising 10 different species in our dataset across all sites. A number of widespread native species associated with Irish and Welsh coastal sites were identified, for example the sponge *Halichondria panicea,*

the polychaete worm *Sabellaria spinulosa*, the barnacle *Semibalanus balanoides* and the ascidian *Ascidiella aspersa*. The full species list of taxonomic assignments and number of reads per site is available in Supplementary Information Table S4.

The taxonomic assignments per site were compared with published lists from RAS for INNS at our study sites (Minchin, 2007; Wood et al. 2015, Table S3), and we were able to identify one known INNS at our study sites. We detected the non-native barnacle *Austrominius modestus* at all sample sites. This species is found throughout Irish and UK waters (Crisp, 1958; O'Riordan, 1996; Minchin, 2007; Wood et al. 2015). We did not detect *D. vexillum* at any of the study sites through metabarcoding, despite the fact that *D. vexillum* colonies are found at Malahide Marina, Carlingford Lough and Holyhead Marina, and this species was detected in our targeted qPCR assay at those sites (Table 1).

Discussion:

In this study, we aimed to determine the potential of eDNA methods (qPCR and metabarcoding) for detection of marine INNS at marina and non-marina coastal sites. We found that qPCR was effective at detecting our target INNS, *D. vexillum*, where it was known to occur, whereas metabarcoding did not detect *D. vexillum*. However, metabarcoding did enable us to detect common marine species found around the coasts of Ireland and the UK as well as the established non-native barnacle *A. modestus*. Thus metabarcoding approaches are potentially useful at detecting multiple INNS in a single analysis and are therefore useful for early detection and as supplementary survey tools. Further, metabarcoding detected the greatest numbers of species at natural sites and the fewest species at closed artificial sites, while intermediate numbers of species were detected at open artificial sites. This eDNA study therefore supports the general conclusion from ecological surveys that biodiversity associated

with artificial shores tends to be lower than on natural rocky shores (Chapman & Bulleri, 2003; Bulleri & Chapman, 2004; Garcia et al. 2007; Vaselli et al. 2008; Pister, 2009).

For targeted detection of *D. vexillum* with qPCR, we developed and tested a novel assay for specific detection of this species in Irish and Welsh seawater samples. We ground-truthed our approach by testing water samples taken from directly beside a *D. vexillum* colony at Malahide Marina. These samples, plus those taken from sites where *D. vexillum* is known to occur (Malahide Marina, Carlingford Lough and Holyhead Marina), all provided positive results, while we did not detect *D. vexillum* at those locations where it has not been previously observed (i.e. Port Oriel, Conwy and Porth Dinllaen). While we did not directly check for the potential for cross species amplification of other species found in the Irish Sea, all positive amplifications were subjected to Sanger sequencing which verified that the amplicon corresponded to *D. vexillium* in all tests. Future studies using the assay outside the sampling area used in the current study should either test for cross amplification with local species or Sanger sequence amplicons to verify the species identity.

In some cases eDNA approaches can be used to give some indication of relative abundance of target species (c.f. Bracken et al. 2018). However, in our case qPCR was not effective at quantifying *D. vexillum* DNA for some of our samples from 2018. This is unsurprising as we sampled a wide area and eDNA may be heterogeneously dispersed in aquatic ecosystems (Takahara et al. 2012; Pilliod et al. 2013). This coupled with the lack of knowledge about tunicate DNA shedding rates in relation to seasonality and environment, may also influence reliable quantitative estimates. However, it is likely that determination of presence versus absence (too low DNA concentrations for detection) is sufficient to achieve the goals of INNS early detection monitoring. We used only publicly available European *D. vexillum*

sequences to design the assay, but it should be possible to design other assays for haplotypes found in other locations (which has previously been done in Australia by Simpson et al. (2017)). As with all other qPCR assays used for eDNA studies, we are limited by the availability of existing samples and sequences taken from public repositories. For this reason, we recommend that representatives for populations of *D. vexillum* throughout its invasive range are sequenced to increase our knowledge of the markers targeted (notably *COI*) for detection of this species using eDNA-based approaches.

Converse to the qPCR results, we did not detect *D. vexillum* through the metabarcoding approach. Primer bias is one possible reason why we failed to detect this species. Primer choice plays an important role in species detection, and it is now accepted that 'general primers' are not truly universal across taxonomic groups (e.g. Piñol et al. 2015; Krehenwinkel et al. 2017). While we attempted to mitigate this by utilising two sets of degenerate primers in a multiplex PCR, our data suggests that these primers preferentially amplified other, potentially more abundant DNA that was present in the complex mix of environmental DNA. It is also possible that our dataset contained sequences originating from *D. vexillum* that may have been lost due to denoising and removal of singleton reads. Another potential reason for lack of *D. vexillum* detection could be removal of sequences found in negative control samples from the analysis. However inspection of those sequences revealed that none were taxonomically assigned to *D. vexillum* or any of the other INNS identified in previous visual (RAS) surveys. It might be that, the competitive nature of PCR, concentrations of *D. vexillum* was too low for metabarcoding to detect the presence and qPCR as it is species specific, could detect lower concentrations of the target species.. A potential approach to increase the detection potential using metbarcoding could be to design and deploy taxon specific primers. However, this approach might require a large number of

primers and it might be, as in our case, that qPCR single species assays are more sensitive for detection of specific INNS.

A limitation of metabarcoding of marine waters identified in this study, and likely problematic in other marine studies as well, was that taxonomic assignment was only possible for a small fraction of the sequences produced. We were unable to assign taxonomy to ~99% of our ASV's, suggesting that there are considerable gaps in the current reference barcode database. We observed that the reference database used in this study (MIDORI_UNIQ; Machida et al. 2017) contained one or more reference sequences for the majority of INNS identified in Irish and Welsh RAS efforts. However, there were no reference sequences for the colonial ascidian *Aplidium cf. glabrum* and the bryozoan *Solidobalanus fallax*. We recommend that efforts should be made to increase the availability of commonly used markers (*COI, 12s,* etc.) for species in the Irish Sea and elsewhere to improve the proportion of ASVs that can be assigned to a species (similar to barcoding initiatives such as the International Barcode of Life (iBOL) project), and particularly for potentially invasive or cryptic species. While there are no currently published studies of eDNA metabarcoding in Irish marine waters, eDNA metabarcoding of seawater samples for INNS detection has been carried out previously in the UK, with sampling sites including Wales (Holman et al. 2019). Though, the specific study site details were not published by Holman et al. (2019) and differences in methodologies precludes direct comparison with our results, their study of INNS at marinas in the UK demonstrated a much higher prevalence of identified INNS. For example, using a 313 bp fragment of *COI*, the authors were able to identify seven out of 21 INNS previously detected during RAS. Further, in an eDNA metabarcoding study of ports in the Bay of Biscay, Borrell et al. (2017) identified three INNS that had been previously recorded at their study sites. However, they utilised two different genetic markers, *COI* and *18s* rDNA, and found that the INNS were detected only in the *18s* rDNA dataset. Grey et al.

(2018) also carried out a metabarcoding study of commercial ports using the same two markers, and found *COI* was more effective for identifying known INNS in the sampling areas, but that 18s rDNA was more effective at detecting unrecorded potential INNS. Combined this suggests that availability of well curated sequence repositories and marker choice plays a particularly important role in detecting INNS and that the use of multiple markers may be required to optimally capture the INNS and overall biodiversity present in the environment.

The findings of our study demonstrate that metabarcoding can be a useful tool for detecting an established INNS but may also miss species that are present in low abundance, that occur in high flushing locations or that remain undetected in a sample due to bias arising from sampling protocols, PCR competition, sequencing errors or primer mismatches. We agree with other authors that metabarcoding can complement conventional survey techniques (Kelly et al. 2017; Djurhuus et al. 2018), rather than act as a direct proxy for visual survey methods. In conclusion, metabarcoding might be less suitable than single species assays for detecting INNS and for future studies and for biosecurity surveillance of specific nuisance INNS like *D. vexillum*, we recommend that the qPCR-based tool presented here can be implemented as it might provide a more sensitive and specific method of detection. However, it should be noted that while qPCR might have higher sensitivity at low DNA concentrations than metabarcoding, too low concentrations could lead to false negative detection. To increase the chances of detection, we recommend sampling close to where the INNS is suspected to occur and to carry out routine temporal sampling to monitor for species presence.

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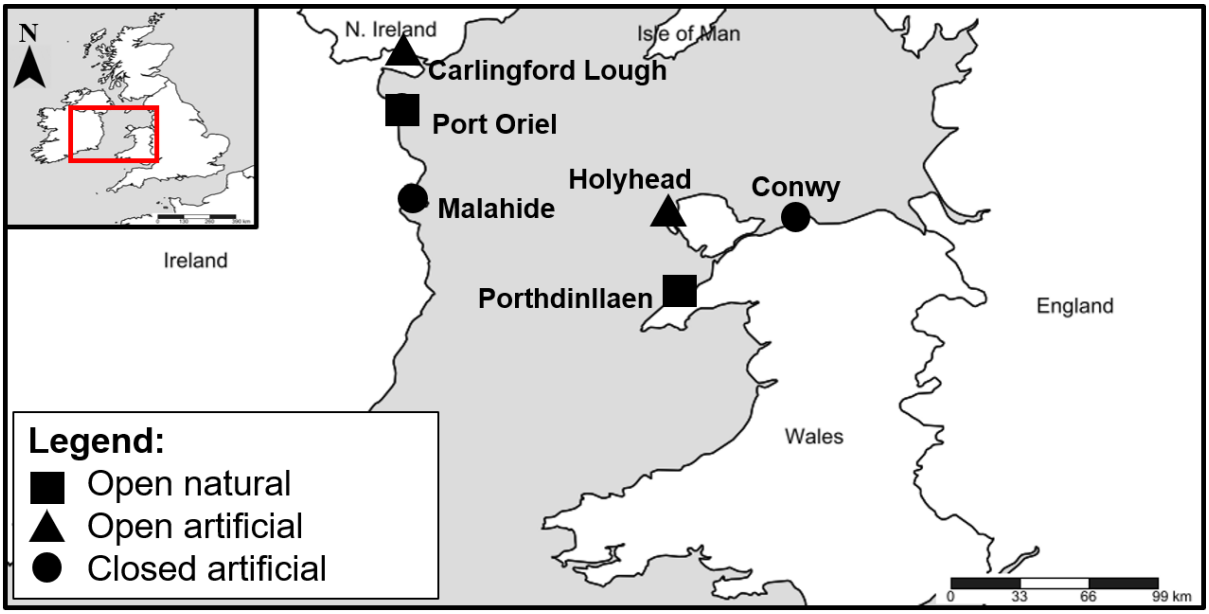
753 

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755 **Figure 1:** Map showing the location of sample sites in Ireland and Wales that were included
756 in this study. Site type is indicated by label shape (see Legend).

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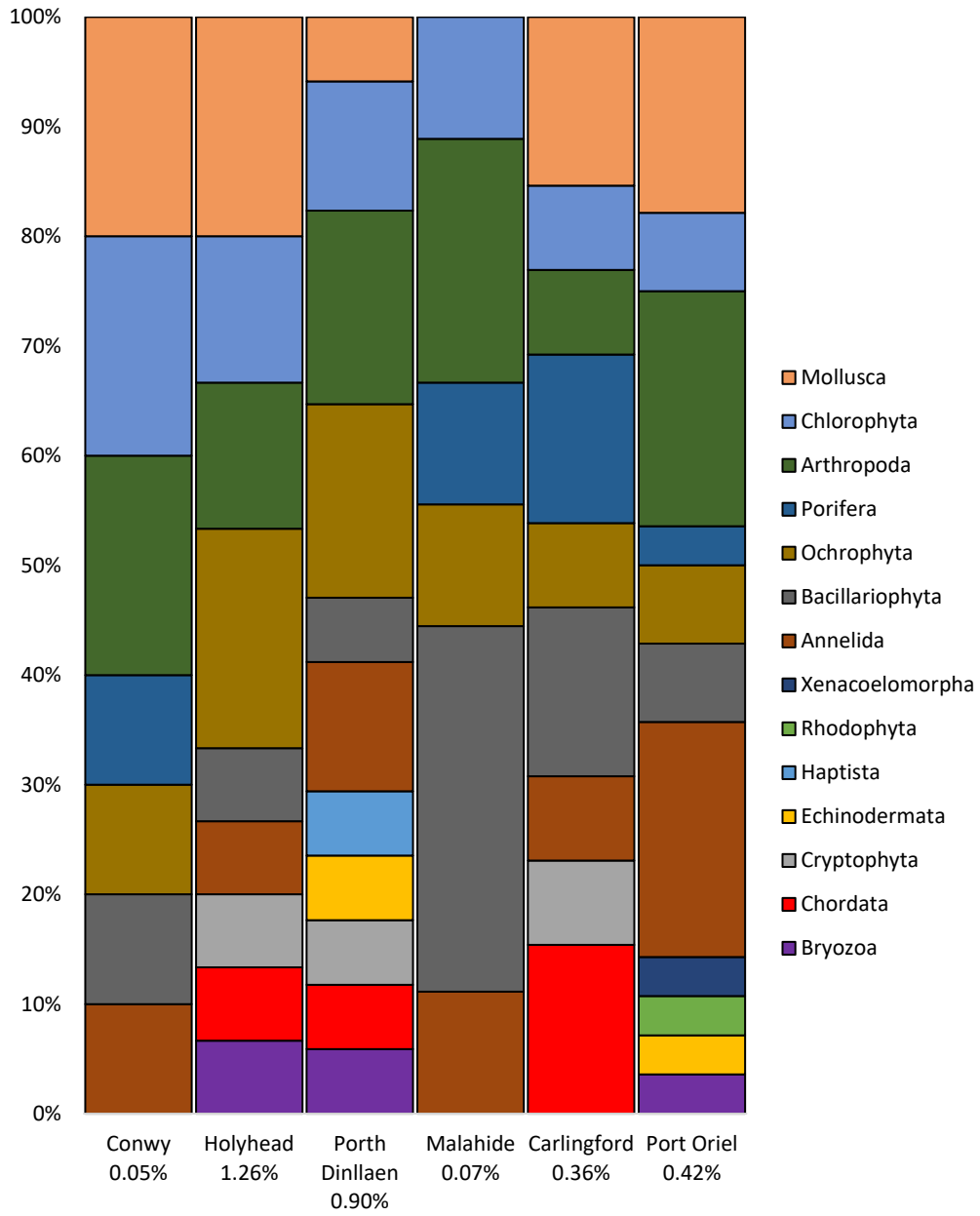

Figure 2: Bar plot showing the proportion of ASV's which were assigned to the taxonomic level of phylum for each sample site. Percentages of sequence reads that could be assigned are listed in the location name.

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