Marine invertebrate voucher specimen sequence typing

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Biodiversity based assessments of aquatic environmental health rely on sampling, sorting and identification of macroinvertebrate communities. In Aotearoa/New Zealand, recent monitoring efforts have been adding environmental DNA (eDNA) sampling to their survey designs, as a complimentary or alternative tool with reduced processing requirements and less need for expert taxonomic knowledge. However, the efficacy of eDNA-based community analyses often depends on the use of libraries of reference DNA sequences to infer the identity of genetic signatures detected in eDNA samples. It is common practice to assemble these libraries from public repositories such as GenBank or the Barcode of Life Database (BoLD), which may be lacking in New Zealand fauna and their available data may be incorrectly identified. Gaps and errors in reference libraries result in uncertainty and imprecision in eDNA taxonomic assignments, reducing their reliability in monitoring efforts. As such, bolstering reference datasets with regionally relevant taxa is an important requirement for effective eDNA applications in environmental health.

The marine environment of New Zealand contains over 10,000 known invertebrate species, making it difficult to assess community diversity at finer taxonomic scales (species and genera) as many taxa have not been DNA-sequenced at genetic barcode loci that are typically employed in eDNA surveys. To overcome this limitation on a regional basis, we used a curated voucher collection of infaunal macroinvertebrate specimens from subtidal sites in Wellington and Porirua harbours. These produced reference DNA sequences at three genetic barcodes, contributing to development of a reference library for use in future eDNA surveys.

A total of 114 taxa were selected for processing, with 82 of these producing viable DNA sequence data from COI, 28S or 16S barcode loci. Sequence data were produced for taxa from eight invertebrate phyla, with most originating from specimens of molluscs, arthropods and annelids. 48 taxa were DNA-sequenced for the first time, significantly expanding the breadth of our regionally focused eDNA reference dataset. While COI and 28S sequencing displayed similar success rates among phyla, the 16S mitochondrial barcode did not amplify in most specimens. The nuclear 28S locus shows promise as a complimentary marker to the commonly used COI locus. Using it as a routine eDNA marker, however, would first require building extensive reference sequence libraries since it has not received as much prior attention as COI.

Based on our findings and the current state of eDNA research, we recommend expanding and developing regionally focused reference sequence sets for use in eDNA surveys, ideally with at least two barcode markers. For the Wellington Region, this would involve DNA-sequencing the remaining 32 unsuccessful invertebrate taxa using other specimens from this region; 21 of these taxa lack sequence data of any kind. Other considerations could include comparisons of sequence data from expert-identified vouchers to pre-existing sequences available in public repositories, processes for screening out contaminant sequences, and sequencing multiple individuals of species to test for population differences in barcode sequences. Finally, we recommend benchmarking the performance of our custom reference library to those that are created from noncurated public repositories, to gain insight into the ramifications of taxonomic gaps and uncertainty in the application of eDNA surveys in the marine environment.

1 Introduction

Assessments of aquatic environments of Aotearoa/New Zealand frequently include measurements of the diversity of invertebrate communities, as an Environmental Health Indicator (*e.g.* the Macroinvertebrate Community Index; MfE 2022) or as a record of natural biodiversity (Taberlet et al. 2018). These assessments require the identification of invertebrate samples to fine-scale taxonomic levels (genera or species) (Hoare et al. 2010), which is a non-trivial exercise for environments that harbour diverse invertebrate communities, such as the marine environment. In New Zealand, over 10,000 invertebrate species are known from the marine environment (Gordon et al. 2010), making it difficult to assess environmental health using a broad-scale community analysis. The burden of macroinvertebrate identification can be overcome using regionally focused collections that have benefitted from rigorous identification of representative taxa. Recent marine surveys in Te Upoke o te Ika/The Greater Wellington Region (GWR) have developed reference collections of benthic macroinvertebrates encountered subtidally in Te Awarua-o-Porirua/Porirua Harbour and Te Whanganui-a-Tara/Wellington Harbour, using expert taxonomic identifications to establish a set of morphological standards against which future surveys may be compared, to assist non-specialist identifications (Cummings et al. 2020a, b).

While community surveys have traditionally used bulk sampling, sorting and identification of physical samples (Stark et al. 2001), such methods are costly when applied in the marine environment since they require the use of snorkelling or SCUBA for sampling plus sorting and identification of many specimens that are distributed across a diverse range of invertebrate phyla. Survey methods that avoid such logistic requirements and which are capable of documenting community diversity *en-masse* are thus an attractive prospect, as cost reductions could allow researchers to provision for more sample replicates or sample on finer timescales. Environmental monitoring efforts in New Zealand have increasingly been exploring and implementing surveillance approaches that include environmental DNA (eDNA) sampling, in part to reduce the costs of traditional labour-intensive survey methods (Zaiko & Steiner 2020), but also to complement traditional methods with alternative data sources (Leduc et al. 2019, Azevedo et al. 2020; Gold et al. 2021). Surveys of eDNA from marine environments typically rely on filtration of seawater samples and/or sampling of benthic sediments, followed by bulk DNA extraction from filters or sediments, then using either amplicon metabarcoding to DNA-sequence a cross-section of community diversity (*e.g.* all metazoans: Nguyen et al. 2020) or species-specific marker detection to target specific taxa (*e.g.* invasive fanworm detection: Wood et al. 2017). Metabarcoding approaches are typically used for community-based assessments of health or biodiversity, whereas targeted eDNA detection is employed in focused biosecurity surveillance programmes or species-specific conservation initiatives. However, many of their limitations are the same: eDNA quantity does not necessarily equate to organismal abundance and type I (false-positive) and II (false-negative) errors are difficult to recognise without complimentary data (Goldberg et al. 2016, Holman et al. 2021).

In order for eDNA surveys to provide a reliable and accurate indication of the presence of species, the DNA sequence data produced by metabarcoding must be matched to reference genetic databases that are taxonomically reliable and contain genetic information for the same taxa found in the sampling area in question. If not, false positives for other species (including non-natives) may result when taxonomic assignment to a 'nearest neighbour' occurs: a sequence cannot be matched to its true source (because it hasn't been sequenced before) and instead is matched to the next most related (but different) species (Claver et al. 2021). A common approach is to assign sequences to higher-level taxonomy (families, genera) when reference libraries are incomplete or of uncertain accuracy (Stat et al. 2017, Locatelli et al. 2020), but this reduces the sensitivity of eDNA approaches

by reducing taxonomic resolution. The ideal solution to low taxonomic coverage in reference libraries is to supplement them with reliable sequence data from specimens that have a high level of confidence in their identification, such as material from taxonomic studies or regional voucher sets that have benefitted from expert attention (Dopheide et al. In Prep).

The current study initiated a reference genetic database for marine invertebrates from Te Whanganui-a-Tara/Wellington Harbour to advance species-level eDNA surveys in areas with similar benthic communities. A curated molecular sequence database was compiled using expertly identified voucher specimens from Wellington and Porirua Harbours, to assist with future validation and implementation of marine eDNA surveys.

The objectives of this study were to:

1. Examine a GWR voucher collection and select up to 138 taxa for genetic analysis,
2. PCR-amplify and DNA-sequence selected voucher subsamples using two barcode markers,
3. Submit curated DNA sequence data to the Barcode of Life Database (BoLD),
4. Examine the efficacy of the resulting dataset for use in future eDNA surveys.

This work supports and provides context to the future use of eDNA sampling in the GWR and will form an important case study for the rest of New Zealand. It will also make a valuable contribution to broader endeavours facilitating the application of eDNA to monitor trends in ocean biodiversity more effectively, which are being undertaken by NIWA.

2 Methods

2.1 Specimen prioritisation, selection, and sampling

Specimens selected for DNA sequencing were chosen from among 630 voucher specimens from Wellington Harbour plus 101 vouchers from Porirua Harbour and Pāuatahanui Inlet, which are archived in the NIWA Invertebrate Collection (see Cummings et al. 2022a and 2022b for collection sites and sampling methods). From these, 118 vouchers were prioritised for this study, representing the breadth of taxonomic diversity among the expert-identified specimen collection. Four of these specimens were excluded due to their small size, thus vouchers would be completely consumed by the DNA extraction process and be unavailable as morphological references for future study. The remaining 114 specimens each represented a unique taxon and were subsampled for DNA extraction and sequencing.

Each selected sample was examined under a dissecting microscope and an approximately 1-5mm^3 tissue subsample was obtained using bleach-sterilised dissecting tools. Tissue subsamples were soaked in 1ml of sterile water to remove excess ethanol and Rose Bengal stain and were stored frozen at -20^{o}C. Specimens were thawed prior to DNA extraction and all water was removed.

2.2 DNA extraction, amplification, and sequencing

DNA extractions from selected invertebrate samples used a DNeasy kit (Qiagen), following the manufacturer's recommended protocol except for the following modifications:

1. All samples were routinely digested in proteinase K overnight in a 56^{o}C rotary incubator at 80 rpm.
2. All samples were eluted with two 40µl volumes of Buffer AE, to maximize DNA concentrations.
3. Small specimens (<5mm) used half volumes of all extraction reagents, except two 40ul elutions were used.

Amplification of genetic loci used primers previously identified in the literature that would produce amplicon sizes suitable for eDNA workflows, based on length restrictions of DNA sequencing platforms typically used for metabarcoding, such as the Illumina MiSeq, and increased detection probabilities for shorter eDNA fragments that are subject to natural environmental degradation. Although the commonly used COI marker was an obvious choice for a primary barcode marker, there was no clear consensus in the recent literature on a second marker for metazoan eDNA applications. Therefore, we trialled two alternative markers that were potentially capable of species-level discrimination and identification: the nuclear 28S rDNA marker and a mitochondrial 16F marker.

Loci were PCR-amplified from genomic DNA extracts using mlCOIintF (GGWACWGGWTGAACWGTWTAYCCYCC) and jgHCO2198 (TANACYTCNGGRTGNCCRAARAAYCA) to amplify ca. 330-350bp of the COI 'barcode' locus (Leray et al. 2013), 28SF_8 (GGGAAAGAAGACCCTGTTGAG) and 28SR_11 (GCTTGGCBGCCACAAGCCAGTTA) to amplify ca. 400-500bp of the nuclear 28S rDNA locus (Machida & Knowlton 2012), and Uni16S_F (TRACYGTGCDAAGGTAGC) and Uni16S_R (YTRRTYCAACATCGAGGTC) to amplify ca. 350-400bp of the mitochondrial 16S rDNA locus (Zhan et al. 2014). PCR reactions were conducted in 25µl total reaction volumes containing 1X MyTaq Mix (Bioline), 600nM of each primer, 12.5µg BSA, and 4-8µl of DNA extract. Thermal profiles used an initial denaturation of 95^{o}C for 3 min, followed by 40 cycles of 95^{o}C

for 15 sec, annealing for 25°C sec, and 72°C for 20 sec, with a final extension of 72°C for 2 min. Reactions used 49°C, 58°C, and 52°C as annealing temperatures for COI, 28S and 16S amplifications, respectively. PCR products were visualised on 1% agarose gels and were treated with ExoSAP-IT (Applied Biosystems) prior to submission for bi-directional Sanger DNA sequencing at a commercial facility (Macrogen Inc.).

Resulting DNA sequences were trimmed and assembled using Geneious Prime v2020.1.1 and chromatograms were visually inspected for quality. The fidelity of sequence data was checked using BLASTn searches, resulting hits were examined for evidence of contamination (best matches to *Homo sapiens* or bacterial, protistan or fungal taxa), and sequences matching non-target phyla were removed. For each of the three marker datasets, alignments of each of the Mollusca, Arthropoda and Annelida (the most abundant taxa) were used to calculate average pairwise identity values, for comparisons of marker variability.

3 Results and Discussion

3.1 Success rates and taxonomic coverage

In total, 114 specimens were extracted, representing 114 unique macroinvertebrate taxa. Of these, 15 failed to amplify at any of the three barcode markers and another 15 only produced low-quality sequences that were discarded. The small size of many invertebrate vouchers plus a need to maintain morphological integrity of vouchers for future use presented challenges for extracting sufficient DNA for amplification and sequencing (Figure 3-1). In addition, we suspected that Rose Bengal staining previously added to voucher specimens to assist with morphological identification was inhibiting DNA extraction or amplification, as seen in previous studies (Fonseca & Fehlauer-Ale 2012; Watanabe et al. 2016).

Figure 3-1: Example of GWR invertebrate voucher specimen used for DNA sequencing. This specimen of *Borniola reniformis* was successfully sequenced at all three barcode markers, despite it being less than 3mm in diameter.

Two samples produced only contaminant sequences and were also discarded. Three other samples produced low-quality contaminant sequences for 28S or 16S markers but produced on-target sequences for COI. These contaminant sequences were discarded while data for COI markers were retained. Contamination of 16S PCRs were attributed to off-target amplification of human DNA (n=2), which produced low quality sequences with high numbers of ambiguous positions. Contamination of 28S PCRs produced low-quality sequences in two instances, which were attributed to dinoflagellates and fungi, and a single high-quality, unambiguous sequence that was attributed to fungi. COI amplifications produced two contaminant sequences in excluded specimens that had no other viable sequence data: a high-quality sequence attributed to fungi and a moderate-quality sequence

attributed to human contamination. There were no obvious marker-related affinities for particular contaminants due to the small sample size, although it was noteworthy that amplification of human DNA contaminants was not observed with the 28S primers. Such contamination risks, although easily identifiable, are difficult to mitigate since universal metazoan primers used for broad scale eDNA diversity assessments do not necessarily exclude non-metazoan eukaryotes (fungi, protozoans) and are designed to include a range of vertebrate taxa, which can include humans (Sepulveda et al. 2020). As seen here, the likelihood of amplification of these non-specific background contaminants is seemingly increased when the availability of target organism DNA is low or non-existent, allowing off-target amplicons with lower primer annealing and amplification efficiency to proliferate during PCR cycling.

Of 114 samples extracted for genomic DNA, 82 (71.9%) produced viable, non-contaminant sequence data at one or more of the three loci (Appendix A), which were submitted to BoLD (JBMFE001-082). Table 3-1 presents a summary of data produced by region and by marker. The 28S locus had the most successful results, followed by COI, then 16S. Multilocus barcodes (two or three markers sequenced) were produced for 50 of the 82 specimens, with 40 specimens sequenced at two loci (COI+28S = 35; COI+16S = 3; 28S+16S = 2) and 10 specimens sequenced at all three loci.

Table 3-1: Summary totals of samples extracted and successfully sequenced for each of three loci. Extracted = counts of specimens for which DNA extractions were performed.

Region	Extracted	COI	28S	16S
Porirua	27	19	18	6
Wellington	87	41	47	11
Totals	114	62	68	19

The taxonomic distribution of sequencing results is shown in Table 3-2 and taxa that produced no viable sequence data from any of the three attempted markers (n=32) are listed in Table 3-3. All included samples of Cnidaria, Priapulida, Echinodermata, Porifera and Platyhelminthes were successfully sequenced at one or more marker loci. The majority of mollusc and arthropod taxa were also successful, whereas more than half the annelid taxa and the sole specimen of Chordata (an unidentified tunicate) were unsuccessful.

Table 3-2: Sequencing results for included invertebrate phyla. For each phylum, the number of samples available for DNA extraction are given, along with the subset of these that produced DNA sequence data for each of the three targeted barcode loci.

Phyla	Extracted	CO1	28S	16S
Annelida	43	13	16	1
Arthropoda	21	15	13	6
Priapulida	1	1	1	0
Chordata	1	0	0	0
Cnidaria	2	2	2	0
Echinodermata	9	9	8	4
Mollusca	35	19	23	6
Platyhelminthes	1	0	1	0
Porifera	1	1	1	0

Table 3-3: List of Wellington and Porirua vouchered taxa lacking sequence data. Taxa are listed for which no sequence data was produced at any of the three targeted loci. Taxon names represent expert identifications, wherever possible. The sole tunicate sample included in this study was not identified.

Annelida:	
Aglaophamus verrilli	*Nicomache nicomache*-A
Aricidea sp.	*Oenonidae*
Asychis trifilosus	*Onuphis aucklandensis*
Axiothella axiothella-B	*Owenia petersenae*
Boccardia syrtis	*Parasabella aberrans*
Euchone pallida	*Pista* sp.
Euclymene insecta	*Prionospio multicristata*
Exogoninae	*Terebellides narribri*
Glycinde trifida	*Urechis novaezealandiae*
Marphysa	
Arthropoda:	
Bemlos? sp. 1	*Liljeborgia* sp. 1
Hippomedon sp.	*Torridoharpinia hurleyi*
Leucon sp.	
Chordata:	
Unidentified tunicate in sandy case	
Mollusca:	
Acanthochitona zelandica	*Roseaplagis artizona*
Amalda australis	*Venerupis largillierti*
Montacuta sp.	*Zemysia globus*
Nozeba emarginata	

Previously, 68 out of the 112 macroinvertebrate taxa listed in Cummings et al. (2022a, b) that were identified to subfamily, genus or species had no sequence data available in GenBank nor BoLD. The 82 taxa which produced sequence data at one or more loci in this study were matched to pre-existing sequence data in GenBank (at any locus – not necessarily just those used here), to produce a list of macroinvertebrate taxa that have herein been sequenced for the first time (Table 3-4). In total, 48 taxa had no prior sequence data available in GenBank (and by extension in the BoLD, which cross-links entries to NCBI-GenBank), reducing the number of unsequenced taxa represented in the voucher collection to 20. The production of new marker data for these 48 taxa represents a significant advancement on the current state of genetic resources for inshore areas in the GWR.

We have also generated complementary sequence data for 33 taxa that already have existing sequence data available. Although it was outside the scope of this study, a comparison to examine concordance of our vouchered sequence data to those already in GenBank or BoLD would provide insight into the prevalence of misidentifications – both within public repositories and among expert-identified collections. There are also an additional 11 taxa that possess pre-existing GenBank sequences, which were not sequenced here; these require closer inspection prior to inclusion in reference datasets, to determine if they originate from reliably identified specimens (*sensu* Locatelli et al. 2020).

Table 3-4: List of newly sequenced macroinvertebrate taxa. List of identified ranks that were sequenced in the current study, for which no previous sequence data is found in GenBank.

Annelida:	
Asychis asychis-B	*Paraprionospio* cf. *pinnata*
Cossura consimilis	*Phylo novazealandiae*
Glycera ?*lamelliformis*	*Pista pegma*
Glycera ovigera	*Prionospio aucklandica*
Labiosthenolepis laevis	*Prionospio yuriel*
Lagis australis	*Pseudopista rostrata*
Nicon aestuariensis	*Timarete anchylochaeta*
Oxydromus angustifrons	
Arthropoda:	
Ampelisca chiltoni	*Jaxea novaezealandiae*
Apseudes novaezealandiae	*Natatolana* cf. *aotearoa*
Bathymedon cf. *neozelanicus*	*Neommatocarcinus huttoni*
Colurostylis whitireia	*Notomithrax*
Halicarcinus whitei	*Upogebia hirtifrons*
Priapulida:	
Priapulopsis australis	
Echinodermata:	
Ophiocentrus novaezealandiae	*Rynkatorpa uncinata*
Pentadactyla longidentis	*Taeniogyrus dendyi*
Mollusca:	
Arthritica bifurca	*Macomona liliana*
Cyclomactra ovata	*Mysella hounselli*
Dosinia greyi	*Neilo australis*
Dosinia lambata	*Neoguraleus murdochi*
Ennucula strangei	*Pratulum pulchellum*
Erycina parva	*Sigapatella tenius*
Hiatula siliquens	*Thracia vitrea*
Leptomya retiaria	*Turbonilla zelandica*
Linucula sp.	
Porifera:	
Suberites cupuloides	

3.2 Barcode marker-specific considerations

A comparison of amplification results for the three barcode markers trialled here (Table 3-2) indicated similar overall and per-phylum rates of success for both COI and 28S. Amplification of 28S produced slightly more successes than COI for annelids and molluscs and was successful for platyhelminths where COI failed, whereas COI amplified two more arthropods and one more echinoderm than 28S. However, these differences were slight and may be due to stochastic errors in

PCR amplification of low-concentration DNA. A comparison of a larger sample size of specimens that also included an examination of the consistency of replicate PCRs would be necessary to distinguish between marker-specific differences and random error effects and this analysis should be included in future efforts.

In comparison to COI and 28S, amplification of 16S had notably lower success rates for all phyla (Table 3-2) and failed to amplify any specimens of Priapulida, Cnidaria, Platyhelminthes and Porifera that were successfully sequenced using the other two markers. The 16S barcode was deemed of lower priority in this study and thus did not undergo the same extent of optimisation and repeat amplification that the other two markers were subjected to (data not shown). The high failure rate may thus be a product of sub-optimal reaction conditions. However, the primer pair used for 16S PCR originated in a study of freshwater invertebrate communities (Zhan et al. 2014) and thus they may not have an affinity for the breadth of marine taxa included here. Zhan et al. (2014) also noted that 16S recovered fewer taxa than 18S amplification, and failed to detect cnidarians, sponges, nematodes and harpacticoid copepods – all of which are also found in marine benthic communities. Although an alternative mitochondrial locus to COI is desirable for corroborating species-level identifications, especially in instances where COI is invariant or uninformative, we recommend exploration of other universal primer sets for 16S, rather than pursuing optimisation of the Zhan et al. (2014) set.

Alignments of sequence data for each of the three most abundant phyla were used to compare the amount of variation observed between the barcode markers (Table 3-5). Patterns of sequence variation were not universal, with COI showing the highest average variation (lowest average identity) for annelids, 16S and 28S having higher variation than COI for arthropods, and 16S and COI having higher variation than 28S for molluscs. These comparisons suggest that the choice of COI as a 'standard' barcode marker for eDNA applications may not always be optimal for fine-scale taxonomic discrimination, particularly for Arthropoda. Incorporating a complementary marker such as 28S into standard eDNA metabarcoding practise may yield higher confidence identifications, although a cost-benefit analysis would first be warranted. Likewise, 16S barcoding could yield improved species diagnoses based on variation levels seen here, but implementation would be hindered by the poor amplification success rates discussed previously, requiring further investigation of alternative primer sets and their inherent taxonomic biases.

Table 3-5: Comparison of variation of marker sequences. For each phylum, the average percent-identity (similarity) is given, along with sample size (in parentheses). No 16S sequence data was generated for Annelida.

Phylum	COI	28S	16S
Annelida	70 (13)	79 (15)	-
Arthropoda	70 (15)	62 (12)	60 (6)
Mollusca	64 (19)	82 (22)	61 (6)

For aquatic invertebrates, eDNA metabarcoding surveys have typically used a COI marker alone (Elbrecht & Leese 2017; Nguyen et al. 2020) or in combination with 18S rDNA (Leduc et al. 2019; Leite et al. 2021) to characterise freshwater and marine communities. While 18S rDNA metabarcoding is generally regarded as too invariant to provide species-level classifications for most metazoans (Drummond et al. 2015), the efficacy of the 28S rDNA subunit has not been explored since early trials (Machida & Knowlton 2012). In combination with mitochondrial markers, a 28S locus has been effective at discriminating species in some cryptic marine invertebrate taxa (Kessel et al. 2022) and shows promise for arthropods and annelids based on the current study. Examples

where other eDNA markers are used for metabarcoding are uncommon, but some studies have targeted subsets of the invertebrate community using mitochondrial 16S primers for crustaceans (Berry et al. 2017) and cephalopods (Peters et al. 2015). While our results also indicate that a taxonomically restricted application of a 16S barcode may be possible, a combination of the mitochondrial COI barcode with a nuclear marker would be preferable since each genome can track speciation events independently (Degnan 1993; Moore 1995) and the mitogenome suffers from low levels of variation in basal marine metazoans such as sponges and cnidarians (Hellberg 2006; Lavrov 2007). Thus, although it is depauperate compared to more common barcoding markers such as COI and 18S, the 28S rDNA gene is worthy of further investigation as a 'universal' species-level marker for metazoans – particularly for marine invertebrates.

4 Conclusions and Recommendations

In summary, we have produced 82 new DNA sequences at up to three loci for common macroinvertebrates in the harbours of the Greater Wellington Region. Of these, 48 taxa had previously not been sequenced before. The number of sequenced taxa for each phylum was roughly proportional to the number of specimens available for each, with annelids, molluscs and arthropods containing most of the sampled taxa and producing most of the DNA sequences. All three markers produced sequenced data but the 16S mitochondrial marker showed poor success rates across all phyla, whereas per-phylum results of COI and 28S sequencing were similar. Instances where few of the taxa produced viable sequences for a given phylum (*e.g.* the Annelida) could be due to either poor affinity for so-called 'universal' PCR primers or to low gDNA availability for amplification due to small specimen sizes.

Based on these findings, we propose the following topics for future consideration and advancement:

Technical Recommendations:

- Further collection and vouchering of marine invertebrates should avoid routine Rose Bengal staining. This could be accomplished by archiving separate unstained subsamples specifically for genetic analysis.
- DNA sequencing results should be carefully screened for evidence of contamination prior to use as a reference.
- 33 taxa sequenced here have pre-existing data in GenBank. Wherever these data cover the same markers, they should be compared to provide an indication of the scale of identification errors in either the voucher collection used here or public sequence repositories such as GenBank and BoLD.
- Repeat sequencing of successful taxa using different specimens could indicate if population-level (*i.e.* intra-specific) variation occurs for barcode markers.

Implementation Recommendations:

- Future sequencing efforts should focus on the remaining 32 taxa collected in the GWR that produced no data in this study. 11 of these have pre-existing data in GenBank, which can be used for comparison and confirmation of sequence fidelity.
- The COI universal barcode region works well for most tested taxa but ideally it should be coupled with a second nuclear marker (potentially 28S) to provide independent corroboration of species-level identifications.
- A database of the sequences produced here plus those available in GenBank should be tested as a taxonomic assignment tool for eDNA sequence data generated from the GWR, in comparison to methods using BoLD or Genbank data alone.
- Expansion of effort to include other regional collections of expertly identified invertebrate specimens in New Zealand. The NIWA Invertebrate Collections contain several such collections that could be prioritised for this purpose, such as the Otago Regional Council State of Environment monitoring samples collected and identified in 2022, which contains 290 specimens representing 82 unique taxa.

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Appendix A

Table A-1: DNA barcoding results for 114 voucher specimens/taxa. For each of the three barcode loci (COI, 28S, 16S), the number of ambiguously determined DNA sequence positions followed by the total DNA sequence length are given. Green indicates sequencing results with ≤2 ambiguities = good sequence quality; yellow indicates 3-9 ambiguities = moderate sequence quality; red indicates ≥10 ambiguities = poor sequence quality.

NIWA No.	COI	28S	16S	Phylum	Full Taxon	Locality	Lot Number	Station ID	Alternate Station name
157758	0/282	7/764		Arthropoda	*Natatolana* cf. *aotearoa*	Wellington Harbour	582	Z18800	WH13B
157761	0/313		0/311	Arthropoda	*Natatolana rossi*	Wellington Harbour	36	Z18792	LB1B
157954	0/313	0/423		Priapulida	*Priapulopsis australis*	Wellington Harbour	261	Z18803	WH1B
158473				Arthropoda	*Bemlos*? sp. 1	Wellington Harbour	596	Z18789	EB2B
158857				Annelida	*Asychis trifilosus*	Wellington Harbour	409	Z18805	WH3B
158858		4/413		Annelida	*Asychis asychis*-B	Porirua Harbour/Pāuatahanui Inlet	636	Z18797	POR1
158862				Annelida	*Euclymene insecta*	Wellington Harbour	247	Z18789	EB2B
158864				Annelida	*Axiothella axiothella*-B	Wellington Harbour	230	Z18791	AQ2B
158869				Annelida	*Nicomache nicomache*-A	Wellington Harbour	133	Z18789	EB2B
158878				Annelida	*Prionospio multicristata*	Wellington Harbour	86	Z18793	LB2B
158888	5/313	0/416		Annelida	*Paraprionospio* cf. *pinnata*	Porirua Harbour/Pāuatahanui Inlet	670	Z18798	POR2
158893	29/236			Annelida	*Prionospio aucklandica*	Porirua Harbour/Pāuatahanui Inlet	716	Z18795	PAH2
158895				Annelida	*Boccardia syrtis*	Wellington Harbour	617	Z18789	EB2B
158903		0/385		Annelida	*Prionospio yuriel*	Wellington Harbour	126	Z18789	EB2B
158913	2/313			Annelida	*Paradoneis lyra*	Porirua Harbour/Pāuatahanui Inlet	675	Z18794	PAH1
158917				Annelida	*Aricidea* sp.	Wellington Harbour	206	Z18791	AQ2B
158927				Annelida	Exogoninae	Porirua Harbour/Pāuatahanui Inlet	683	Z18794	PAH1
158934	0/313	1/425		Annelida	*Eulalia* sp.	Wellington Harbour	289	Z18803	WH1B
158939		4/214		Annelida	*Armandia maculata*	Wellington Harbour	112	Z18789	EB2B
158940		6/238		Annelida	*Lagis australis*	Wellington Harbour	168	Z18790	AQ1B
158942			51/341	Annelida	*Timarete anchylochaeta*	Porirua Harbour/Pāuatahanui Inlet	710	Z18795	PAH2
158946	0/313	7/366		Annelida	*Aphelochaeta* sp.	Wellington Harbour	15	Z18792	LB1B
159110	2/313		0/318	Arthropoda	*Apseudes novaezealandiae*	Porirua Harbour/Pāuatahanui Inlet	653	Z18797	POR1
159116				Annelida	*Glycinde trifida*	Wellington Harbour	625	Z18789	EB2B
159117	0/313			Annelida	*Glycera ovigera*	Wellington Harbour	624	Z18789	EB2B
159120		0/376		Annelida	*Hemipodia simplex*	Wellington Harbour	240	Z18789	EB2B
159130		2/352		Annelida	*Glycera* ?*lamelliformis*	Wellington Harbour	11	Z18792	LB1B
159137		35/344		Annelida	*Oxydromus angustifrons*	Wellington Harbour	323	Z18799	WH10B
159154		12/324		Annelida	Lumbrineridae	Wellington Harbour	244	Z18789	EB2B
159162				Annelida	Oenonidae	Wellington Harbour	266	Z18803	WH1B
159179	0/313	6/351		Annelida	*Cossura consimilis*	Wellington Harbour	13	Z18792	LB1B
159228	0/313	1/415		Annelida	*Labiosthenolepis laevis*	Wellington Harbour	256	Z18803	WH1B
159247				Annelida	*Aglaophamus verrilli*	Wellington Harbour	10	Z18792	LB1B
159248		0/365		Annelida	*Serpula* sp.	Porirua Harbour/Pāuatahanui Inlet	728	Z18796	PAH3
159252				Annelida	*Terebellides narribri*	Wellington Harbour	116	Z18789	EB2B
159253				Annelida	*Marphysa* sp.	Wellington Harbour	604	Z18789	EB2B
159254				Annelida	*Owenia petersenae*	Wellington Harbour	120	Z18789	EB2B
159255				Annelida	*Onuphis aucklandensis*	Wellington Harbour	284	Z18803	WH1B
159256				Annelida	*Euchone pallida*	Wellington Harbour	614	Z18789	EB2B
159259				Annelida	*Parasabella aberrans*	Wellington Harbour	618	Z18789	EB2B
159263	0/313	0/436		Annelida	*Nicon aestuariensis*	Porirua Harbour/Pāuatahanui Inlet	639	Z18797	POR1
159274	0/313			Annelida	*Pseudopista rostrata*	Porirua Harbour/Pāuatahanui Inlet	666	Z18798	POR2
159276	2/310			Annelida	*Phylo novazealandiae*	Wellington Harbour	219	Z18791	AQ2B
159281		156/478		Annelida	*Pista pegma*	Wellington Harbour	619	Z18789	EB2B
159285				Annelida	*Pista* sp.	Wellington Harbour	607	Z18789	EB2B
159310	1/313			Annelida	*Harmothoe* sp.	Wellington Harbour	29	Z18792	LB1B
159318	5/313			Annelida	*Lepidonotus* sp.	Wellington Harbour	408	Z18805	WH3B
159321	0/312	0/413	0/343	Mollusca	*Struthiolaria papulosa*	Wellington Harbour	203	Z18791	AQ2B
159323	0/313	3/426	0/307	Mollusca	*Cyclomactra ovata*	Porirua Harbour/Pāuatahanui Inlet	641	Z18797	POR1
159324			0/356	Mollusca	*Maoricolpus roseus*	Wellington Harbour	111	Z18789	EB2B
159325	0/313	1/462	0/359	Echinodermata	*Paracaudina chilensis*	Porirua Harbour/Pāuatahanui Inlet	682	Z18794	PAH1
159327	20/313			Mollusca	*Dosinia greyi*	Wellington Harbour	217	Z18791	AQ2B
159330	0/313	0/502	0/367	Arthropoda	*Upogebia hirtifrons*	Wellington Harbour	600	Z18789	EB2B
159331	2/312	0/511	0/360	Arthropoda	*Hemiplax hirtipes*	Porirua Harbour/Pāuatahanui Inlet	708	Z18794	PAH1
159332	0/313	2/425		Echinodermata	*Rynkatorpa uncinata*	Wellington Harbour	441	Z18808	WH9B
159333	0/313	0/543		Echinodermata	*Pentadactyla longidentis*	Wellington Harbour	361	Z18808	WH9B
159338	0/313	0/528	0/361	Arthropoda	*Neommatocarcinus huttoni*	Wellington Harbour	178	Z18790	AQ1B
159342	0/312	2/537	0/351	Arthropoda	*Jaxea novaezealandiae*	Wellington Harbour	574	Z18800	WH13B
159346	0/313	49/288		Arthropoda	*Notomithrax* sp.	Wellington Harbour	410	Z18805	WH3B
159352	4/75		0/418	Echinodermata	*Echinocardium cordatum*	Wellington Harbour	5	Z18792	LB1B
159357	0/313	0/472		Arthropoda	*Halicarcinus varius*	Porirua Harbour/Pāuatahanui Inlet	668		POR02B
159362	4/313	11/429		Arthropoda	*Halicarcinus whitei*	Porirua Harbour/Pāuatahanui Inlet	703		PAH01B
159363	11/313			Mollusca	*Dosinia lambata*	Wellington Harbour	109		EB02B
159365				Mollusca	*Amalda australis*	Porirua Harbour/Pāuatahanui Inlet	729		PAH03B

NIWA No.	COI	28S	16S	Phylum	Full Taxon	Locality	Lot Number	Station ID	Alternate Station name
159395	1/313	0/426		Echinodermata	*Taeniogyrus dendyi*	Wellington Harbour	612		EB02B
159396	6/277	0/415		Cnidaria	*Edwardsia* sp.	Porirua Harbour/Pāuatahanui Inlet	713		PAH02B
159458		12/309		Arthropoda	*Colurostylis whitireia*	Porirua Harbour/Pāuatahanui Inlet	699		PAH01B
159465				Arthropoda	*Torridoharpinia hurleyi*	Wellington Harbour	474		WH02B
159477	24/128			Arthropoda	*Leptostylis* sp.	Wellington Harbour	286		WH01B
159478				Arthropoda	*Liljeborgia* sp. 1	Wellington Harbour	595		EB02B
159479				Arthropoda	*Hippomedon* sp.	Porirua Harbour/Pāuatahanui Inlet	667		POR02B
159482	0/313	0/428		Echinodermata	*Amphipholis squamata*	Wellington Harbour	136		EB02B
159483	3/228	0/418		Echinodermata	*Ophiocentrus novaezealandiae*	Porirua Harbour/Pāuatahanui Inlet	705		PAH01B
159484	0/245	1/421	0/327	Echinodermata	*Amphiuridae* indet. (Juvenile)	Wellington Harbour	552		WH013B
159487	7/132	0/423		Arthropoda	Harpacticoid copepod	Wellington Harbour	587		WH017B
159489	4/278	0/435		Cnidaria	*Anthopleura aureoradiata*	Porirua Harbour/Pāuatahanui Inlet	692		PAH01B
159562				Chordata	Tunicate in sandy case	Wellington Harbour	611		EB02B
159563				Mollusca	*Acanthochitona zelandica*	Wellington Harbour	608		EB02B
159569	0/313	1/556		Arthropoda	*Bathymedon* cf *neozelanicus*	Wellington Harbour	207		AQ02B
159583				Arthropoda	*Leucon* sp.	Wellington Harbour	189		AQ02B
159596	3/239	0/521		Arthropoda	*Ampelisca chiltoni*	Wellington Harbour	508		WH05B
159603	13/192	0/521		Arthropoda	*Ampelisca* sp.	Wellington Harbour	308		AQ02B
159606	0/313	0/431		Mollusca	*Leptomya retiaria*	Wellington Harbour	236		EB02B
159610	0/313	0/419		Mollusca	*Neoguraleus murdochi*	Wellington Harbour	627		EB02B
159611		13/338		Mollusca	*Dosinia* sp. juvenile	Wellington Harbour	273		WH01B
159616	0/313	0/428		Mollusca	*Xymene plebeius*	Porirua Harbour/Pāuatahanui Inlet	695		PAH01B
159620		1/414		Mollusca	*Sigapatella tenius*	Wellington Harbour	250		EB02B
159621		0/432	0/308	Mollusca	*Scintillona* sp.	Wellington Harbour	107		EB02B
159624		1/408		Mollusca	*Philine auriformis*	Wellington Harbour	411		WH03B
159640		0/411		Platyhelminthes	Stylochidae	Wellington Harbour	265		WH01B
159657	0/313	0/430	0/305	Mollusca	*Arthritica bifurca*	Porirua Harbour/Pāuatahanui Inlet	631		POR01B
159670		0/390	0/334	Mollusca	*Neilo australis*	Wellington Harbour	309		WH010B
159684	0/242			Mollusca	*Thracia vitrea*	Wellington Harbour	581		WH013B
159690				Mollusca	*Zemysia globus*	Wellington Harbour	546		WH05B
159691	11/241	3/386		Mollusca	*Corbula zelandica*	Wellington Harbour	599		EB02B
159696				Mollusca	*Montacuta* sp.	Wellington Harbour	362		WH09B
159698		0/430		Mollusca	*Erycina parva*	Wellington Harbour	79		LB02B
159702		0/410		Mollusca	*Macomona liliana*	Porirua Harbour/Pāuatahanui Inlet	673		PAH01B
159707	0/313	0/426		Mollusca	*Tawera spissa*	Wellington Harbour	108		EB02B
159709	0/313			Mollusca	*Austrovenus stutchburyi*	Porirua Harbour/Pāuatahanui Inlet	658		POR01B
159712	4/314	0/394		Mollusca	*Hiatula siliquens*	Wellington Harbour	598		EB02B
159717	0/313	0/440		Mollusca	*Borniola reniformis*	Wellington Harbour	458		WH04B
159722		14/330		Mollusca	*Linucula* sp. 1 (Spencer, 2009)	Wellington Harbour	615		EB02B
159736	0/313	1/414		Mollusca	*Turbonilla zelandica*	Porirua Harbour/Pāuatahanui Inlet	711		PAH02B
159741	0/313	0/445		Mollusca	*Mysella hounselli*	Wellington Harbour	589		WH017B
159743				Mollusca	*Nozeba emarginata*	Wellington Harbour	543		WH05B
159745				Mollusca	*Roseaplagis artizona*	Wellington Harbour	610		EB02B
159758	0/313	57/933		Mollusca	*Ennucula strangei*	Wellington Harbour	54		LB02B
159759	0/313	0/441		Mollusca	*Theora lubrica*	Porirua Harbour/Pāuatahanui Inlet	672		PAH01B
159788	0/313	0/428		Mollusca	*Pratulum pulchellum*	Wellington Harbour	567		WH013B
159789				Mollusca	*Venerupis largillierti*	Wellington Harbour	105		EB02B
159931	3/244	0/426	0/332	Echinodermata	*Amphiura rosea*	Wellington Harbour	21		LB01B
159940				Annelida	*Urechis novaezealandiae*	Wellington Harbour	27		LB01B
159943	0/313	3/400		Porifera	*Suberites cupuloides* Bergquist, 1961	Wellington Harbour	613		EB02B