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Metabarcoding improves detection of eukaryotes from early biofouling communities: implications for pest monitoring and pathway management

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ABSTRACT

In this experimental study the patterns in early marine biofouling communities and possible implications for surveillance and environmental management were explored using metabarcoding, *viz.* 18S ribosomal RNA gene barcoding in combination with high-throughput sequencing. The community structure of eukaryotic assemblages and the patterns of initial succession were assessed from settlement plates deployed in a busy port for one, five and 15 days. The metabarcoding results were verified with traditional morphological identification of taxa from selected experimental plates. Metabarcoding analysis identified > 400 taxa at a comparatively low taxonomic level and morphological analysis resulted in the detection of 25 taxa at varying levels of resolution. Despite the differences in resolution, data from both methods were consistent at high taxonomic levels and similar patterns in community shifts were observed. A high percentage of sequences belonging to genera known to contain non-indigenous species (NIS) were detected after exposure for only one day.

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Introduction

In marine environments, submerged material provides a substratum for the rapid formation of biofilms which comprise organic matter, bacteria, and microalgae, and develop into mature biofouling communities over time (Cooksey & Wigglesworth-Cooksey 1995). Extensive biofouling on underwater structures can have significant economic consequences on many maritime industries. For example, it can impact aquaculture through overgrowing farming facilities and stock species, reducing the yield by competing for resources with cultured organisms, producing toxic secondary metabolites, or reducing oxygen supply (Fitridge et al. 2012). In ports and marinas biofouling can result in considerable maintenance costs for infrastructure, such as pontoons, piling, navigation buoys, and resident vessels (Schultz 2007; Adams et al. 2011). In coastal areas, biofouling communities often include a large proportion of non-indigenous species (NIS) and hull fouling is acknowledged as one of the most important mechanisms for their dispersal (Hewitt et al. 2004; Hayden et al. 2009). Established populations of NIS in transport hubs such as ports and marinas can then serve as reservoirs or 'stepping stones' for subsequent secondary introductions (Floerl et al. 2009).

To date there are no global regulatory mechanisms aimed at reducing biofouling-related risks of NIS introduction, except for some advisory documents (eg IMO 2011). However, there are national legislative initiatives that are starting to address this issue; for instance, New Zealand's Craft Risk Management Standard (CRMS; MPI 2014). This is currently a voluntary standard that is anticipated to become mandatory in 2018. This standard sets thresholds for management of biofouling risks associated with vessels and differentiates between 'short-stay vessels' (those remaining in New Zealand for 20 days or less) and 'long-stay vessels' (those remaining for 21 days or longer). The underlying concept of this differentiation is the assumption that during a short-term stay it is unlikely that a mature, reproductively active biofouling community develops on visiting vessels. Consequently, a low risk of NIS introduction is attributed to those vessels. During longer stays, even minimal hull fouling might become a source of incursion by maturing and developing

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reproductive stages. Such vessels are seen to pose a higher risk of pest transfer and are therefore treated in a more stringent way (Ashton et al. 2006; Georgiades & Kluza 2014).

If an incursion occurs, the efficacy of pest control and success of response measures can be greatly facilitated through the early detection (Myers et al. 2000). Currently the methodology used to detect NIS at the border and post-border is reliant on morphological expertise, which is declining globally, and as a result accurate identification of organisms at all taxonomic levels and life stages is increasingly rare (Hopkins & Freckleton 2002; Costello et al. 2013). This may restrict the timely detection and valid identification of potential pest species, even if an adequate surveillance program is in place.

Rapidly advancing molecular techniques provide promising tools for species identification from environmental samples. For instance, gene barcoding in combination with high-throughput sequencing (HTS), also known as metabarcoding, allows effective community-wide biodiversity assessments, providing presence/absence or semi-quantitative data on microbial, protists and other eukaryotic assemblages (Taberlet et al. 2012; Wood et al. 2013; de Vargas et al. 2015). Metabarcoding has already been successfully used for describing marine communities from sediments (Chariton et al. 2010; Pochon et al. 2013), biofouling (Leary et al. 2014; Muthukrishnan et al. 2014; Xue et al. 2014; Pochon, Zaiko et al. 2015; Lawes et al. 2016) and ballast water (Zaiko et al. 2015). These techniques may help to fill important knowledge gaps around early biofouling communities and associated risks of biological invasions.

In this experimental study metabarcoding was used to identify the taxonomic composition of early biofouling communities on settlement plates deployed within a port area. The performance of the method was assessed in comparison to traditional morphological identification. The wider implications of metabarcoding for NIS monitoring and pathway management were also addressed by evaluating its capacity to distinguish patterns in community composition and its ability to detect potential pests in early eukaryotic assemblages.

Materials and methods

Experimental set-up and sample collection

This study is an extension of a recruitment experiment performed in one of New Zealand's major shipping ports, located in Lyttelton, South Island, New Zealand (Figure 1). The experimental set-up and sample collection described by Schimanski (2015) were used. A brief summary is provided here.

During the experiment, a total of 184 settlement plates (15 × 15 cm, made of light gray 4 mm thick polyvinyl chloride, PVC) were attached to a larger PVC backing plates and deployed vertically in two locations (0.5–1.0 m below the waterline); 92 settlement plates at each location over two experimental rounds. Sampling sites were located 500 m from each other in a port characterized by an active tidal-driven water circulation with an average residence time of about two days (Hart 2004). Therefore, the environmental conditions at both sites were assumed to be relatively uniform and any observed differences in biofouling patterns would reflect the small-scale recruitment heterogeneity within the study area.

Based on earlier studies (eg Dobretsov 2009; Pochon, Zaiko et al. 2015) and pre-experimental trials (unpublished data), it was expected that the heterogeneity in early biofouling communities might be inversely related to the time of exposure. To account for this, a stratified sampling design was applied with sampling effort (n) reducing between the three exposure treatments: Day 1 (n=116 samples), Day 5 (n=40 samples), and Day 15 (n=28 samples). It was assumed that dividing the samples into relatively homogenous groups based on the exposure treatment and sampling on earlier days more heavily would increase the overall precision of the experiment. All exposure treatments fell within the period defined for short-stay vessels within New Zealand's CRMS (MPI 2014). Two experimental rounds (n=46 per round × location) were undertaken during the austral summer (2–17 January 2013 and 11–26 February 2013) to assess the temporal variability in recruitment patterns over the peak spawning season in temperate coastal waters. The water temperature at the time of experiments ranged between 15 and 20°C. Replicate plates were randomly selected and detached one, five and 15 days after deployment. These were placed into individually sealed plastic bags, transported to the laboratory in an insulated container and stored at –20°C until further processing.

DNA extractions and high-throughput sequencing

Sterilized sponges (Whirl-pak™, Speci-sponges™, Nasco, Fort Atkinson, WI, USA) were used to collect biofilm samples by thoroughly rubbing all material from the surface of each thawed plate (Pochon, Zaiko et al. 2015). The sponge and collected material were isolated into individually sealed sterile plastic bags provided by the same manufacturer. RNA/DNA free water (40 ml; Life Technologies, Carlsbad, CA, USA) was added to the plastic bags, and the sponges were macerated using a Colworth 400 laboratory stomacher (2 min; Seward, Worthing, UK). Excess liquid was squeezed from the sponges and all liquid and

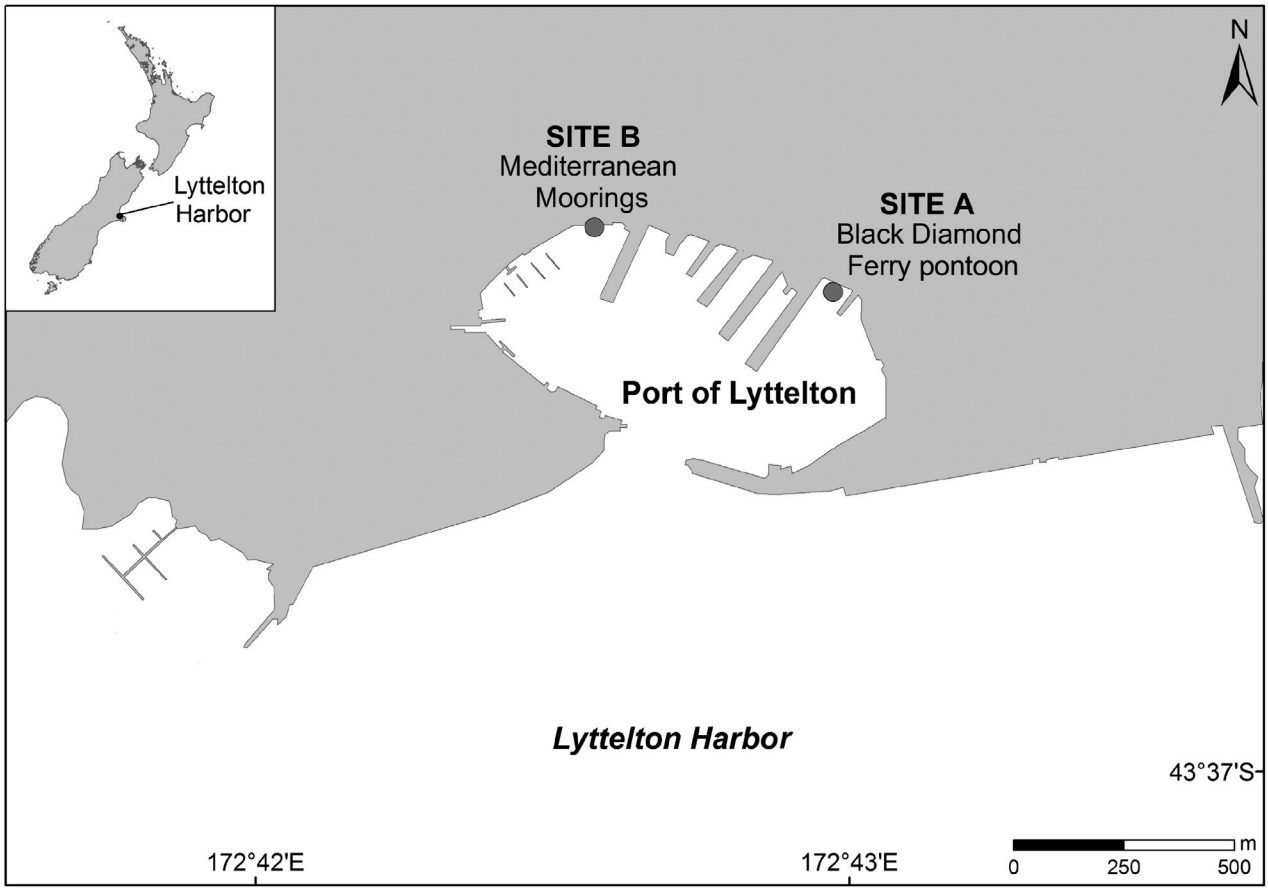

Figure 1. Location of the two experimental sites within the Port of Lyttelton, New Zealand.

material transferred to sterile 50 ml tubes. The suspension was pelleted by centrifugation (3,000 × *g*, 15 min). The supernatant was discarded and DNA extracted from the pellet using a Power Biofilm® DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) following the manufacturer's instructions. The quantity and quality of extracted DNA samples were assessed using a NanoPhotometer (Implen, Munich, Germany). In total, 184 DNA extractions were conducted.

A two-step tailed PCR amplicon procedure (Sreemanta & Honghua 2002) was employed to generate an Illumina MiSeq™ library. The universal primers Uni18SF: AGGGCAAKYCTGGTGCCAGC and Uni18SR: GRCGGTA-TCTRATCGYCTT (Zhan et al. 2013) were used to amplify the eukaryotic V4 region of the nuclear small subunit ribosomal DNA (18S rRNA) gene. The primers were modified to include Illumina™ overhang adaptors as described in Kozich et al. (2013). PCR amplifications were undertaken on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) in a total volume of 30 µl using AmpliTaq Gold® 360 PCR Master Mix (Life Technologies), 1 µl of each primers and 1 µl of template DNA. Reaction cycling conditions were: 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s, and a final extension of 72°C for 8 min.

For internal quality control, DNA samples from three previously isolated marine species, *viz*. *Sabella spallanzanii*, *Ciona savignyi* and *Perna perna* (Pochon et al. 2013) were PCR amplified following the same protocol as described above. After purification and quantification these were combined at three distinct concentrations (Control 1: equimolar, Control 2: 10:5:1 and Control 3: 1:5:10). Two replicates of each were incorporated into the HTS library.

All amplicons were diluted to a concentration of 1 ng μl^{-1} and sent to New Zealand Genomics Limited (NZGL), University of Auckland, for library preparation. Sequencing adapters and sample-specific indices were added to each amplicon *via* a second round of PCR using the Nextera™ Index kit (Illumina™). Amplicons were pooled into a single library and paired-end sequences (2 × 250) generated on a MiSeq instrument using the TruSeq™ SBS kit (Illumina™). Sequence data were automatically demultiplexed using MiSeq Reporter (v2) (Illumina, http://www.illumina.com/systems/miseq/software/miseq-reporter.html), and forward and reverse reads assigned to samples.

Morpho-taxonomic analysis of settled organisms

To validate the metabarcoding results with a traditional baseline technique, five plates were randomly selected from each treatment (total n=60) and visually examined, before freezing, using a Nikon SMZ-IB (Nikon Metrology, Alzenau, Germany) stereo microscope at 35 × magnification for initial screening. Plates revealing the presence of any organisms were then processed with 70 × and/or 100 × magnification for further identification. All organisms were photographed using a Leica M205C stereo microscope with a DFC 295 (3MP) digital camera and identified to the lowest taxonomic level possible using taxonomy keys (Gordon & Mawatari 1992; Hayward & Ryland 1995; Bullard & Whitlatch 2004). For each identified taxon, abundance was assessed by counting the number of recruits (individual or a colony) on plates.

Bioinformatic analyses

Bioinformatic analysis of metabarcoding data was performed using MOTHUR v.1.34.4 (Schloss et al. 2009). Forward and reverse paired-end sequences were assembled independently for each sample. The bioinformatics pipeline and analysis procedure described in Kozich et al. (2013) was applied as described below.

Overlapped raw sequence reads were de-noised, trimmed and filtered prior to downstream analyses. The retained sequences were de-replicated into unique sequences and aligned against the reference SILVA database for eukaryotic ribosomal RNA sequences (Quast et al. 2013). PCR-mediated recombination in amplification products (ie chimeras) were identified and removed from the dataset using the UCHIME algorithm (Edgar et al. 2011) both in *de novo* and dataset modes.

Sequence reads from each sample were clustered into operational taxonomic units (OTUs) at 97% similarity. In order to reduce the potential for introducing artefactual singletons in the dataset (eg, Brown et al. 2015), OTUs represented by a single sequence were discarded. The remaining OTUs were taxonomically assigned using the Protist Ribosomal 2 (PR2) database (Guillou et al. 2013). For taxonomic classification, the Wang method was applied with the minimum bootstrap value set at 97% (Wang et al. 2007). Additionally, taxa represented by < 10 sequences over the entire dataset were removed to minimize the potential introduction of false signals resulting from incidental (extracellular) DNA commonly found in the environment (eg Pawlowski et al. 2014). Taxonomic assignments were verified against the World Register of Marine Species, AlgaeBase, Encyclopedia of Life, Integrated Taxonomic Information System and New Zealand Organisms Register and validated by taxonomic experts.

After stringent screening of the taxonomic data, all putative sequences were discarded from 'external sources' (ie, assigned to terrestrial taxa). The retained dataset of assigned taxa and records of the number of sequences per sample were used for biodiversity assessment and statistical analyses.

Statistical analyses

Analyses were undertaken using the PRIMER 7 software package (PRIMER-E Ltd, Ivybridge, UK) and the R v3 statistical computing environment (R-project 2014). The hierarchical data browser tool Databurst for Excel (Appz, London, UK) was used for visualizing the taxonomic composition from metabarcoding and morphological analyses. In the analyses, the following factors (explanatory variables) were considered: (*i*) experimental round; (*ii*) location within the port, and (*iii*) exposure period. All factors were fully crossed and appropriately replicated.

The untransformed metabarcoding data (number of reads per OTU per sample) were used to generate OTU rarefaction curves for each treatment using the R software 'vegan' package (Oksanen et al. 2014). Differences in biofouling community composition between treatments were assessed using distance-based permutational analysis (PERMANOVA, Anderson 2001). In the PERMANOVA design, exposure was included as a fixed factor, and round and location were treated as random factors. Given the limited taxonomic resolution of the morphologically analyzed samples, only metabarcoding data were included in these analyses. Analyses were based on Bray–Curtis similarities using fourth-root transformed percentage and presence–absence data. This approach was considered appropriate to account for possible biases associated with the limited quantification capacity of metabarcoding (Kelly et al. 2014).

Shade plots were used to visually display common patterns of change in the relative abundance (represented by percentage of sequence reads) for most important taxa (those contributing more than 10% to the total number of sequence reads within a sample). The proportional contribution of putative pest species in the dataset was assessed by selecting taxa listed as non-indigenous, cryptogenic or pest organisms in New Zealand inventories (eg Marine Biosecurity Porthole database, http://marinebiosecurity.org.nz/) and published resources (Hayward 1997; Cranfield et al. 1998; Primo & Vázquez 2008). To minimize the risk of marker resolution-related bias, the contribution of putative pest species was analyzed at the genera level. The effect of the three factors on the relative

abundance of those genera (percentage of sequence reads) was assessed using the non-parametric factorial analysis of variance (ANOVA) implemented in ARTool R-package (ARTool, Wobbrock et al. 2011).

Results

DNA quality and high-throughput sequencing output

DNA yields varied from undetectable to 60.5 ng μ^{-1}, and nanophotometer absorbance ratios 260/280 ranged between 0 and 2. The quantity and quality of extracted DNA generally increased with exposure time. Conversely, the variance in DNA quality (absorbance ratio) decreased over time for each treatment (Figure S1 in Supplemental material). The V4 region of 18S rRNA gene was successfully amplified from 105 samples. The average rate of successful amplifications was 34, 90 and 96% for plates deployed for one, five and 15 days respectively.

Raw sequencing data from the 105 positive PCR amplicons comprised 5,481,689 sequences. The stringent sequence filtering parameters resulted in the removal of 65.7% reads, yielding 1,879,884 high-quality reads with an average length of 425 bp for downstream analyses. Two samples (s52: Round 1, Site A, Day 5, and s80: Round 2, Site B, Day 5) produced fewer than 1,000 high-quality sequences (729 and 18, respectively), and were removed from subsequent analyses. The number of high quality useable sequences per treatment is summarized in Table S1.

Taxonomic assignment of high-throughput sequence data

Rarefaction curves indicated that all samples maintained in the final analysis were adequately sequenced (Figure S2). The majority (1,601,396; 99.4%) of sequences were taxonomically assigned against the reference database (to a supergroup or lower taxonomical level). Of those, 8,527 (0.5%) sequences were attributed to putative 'external source' taxa (eg terrestrial plants, lichens, mites, and mammals). After discarding those taxa and taxa represented by < 10 sequences over the entire dataset (0.06%), the final assignment comprised seven supergroups, 33 phyla, 73 classes, 132 orders, 195 families, 240 genera and 182 species (Table S2). The majority of sequences were assigned to Metazoa (57%) and Archaeplastida (34%; Figure 2A and B).

The taxonomic assignment of the internal control samples confirmed the robustness of the sequencing and analytical pipeline. Of the 154,756 high-quality sequences produced, > 99.9% were assigned to the target taxa (Figure 3), although the V4 sequences belonging to *P. perna* could only be assigned at the family level. The proportions of sequences were consistent with those expected.

Morphological identification

Overall, the taxonomic diversity and resolution based on morphological identification were markedly lower compared to those obtained with metabarcoding. Visual inspection of plates led to the identification of 25 taxa belonging to 11 phyla, 10 classes, five orders and seven families. Approximately 1.5% of detected recruits were impossible to unambiguously identify, and therefore remained 'unclassified'. Most (~80%) of the organisms were at the early developmental stage and were assigned to a coarse taxonomic resolution, ie Family, Class or Phylum. Only six taxa were identified at the genus level with reasonable confidence: *Bugula*, *Ciona*, *Diplosoma*, *Molgula*, *Spirorbis* and *Watersipora* (Table S3). Consistent with metabarcoding, the majority of recruits were assigned to Metazoa (68%) and Archaeplastida (25%; Figure 2C and D).

Shifts in biofouling communities

At the highest taxonomic level, consistent shifts in biofouling community composition were revealed using metabarcoding at both study sites over the two experimental rounds (Figure 4A). During the first experimental round, communities changed from animal-dominated (average number of Metazoa sequences: 65%) on Day 1 after deployment, to algae-dominated (average number of Archaeplastida sequences: 66%) on Day 15. During the second round, communities remained dominated by metazoans (60–79% of sequence reads) across all exposure treatments.

The morphologically analyzed samples revealed similar patterns, with an increase in algae during the first experimental round (Figure 4B). On all plates, however, the communities were highly dominated by metazoans (shifting from 96% of total recruit abundance on the first day to 78% on the 15th day). During the second round, the number of observed Alveolata (represented by unclassified ciliates and diatoms) increased with the exposure time from 0.5 to 14% of recruit abundance.

Based on the metabarcoding percentage abundance data, community structure was mostly affected by exposure, marginally by the experimental round and their interaction (PERMANOVA, $p<0.01$, $p=0.08$ and $p=0.05$ respectively). PERMANOVA performed on presence–absence data confirmed the importance of exposure ($p<0.01$); however, experimental round was not significant ($p=0.2$) and their interaction had a marginal effect ($p=0.08$).

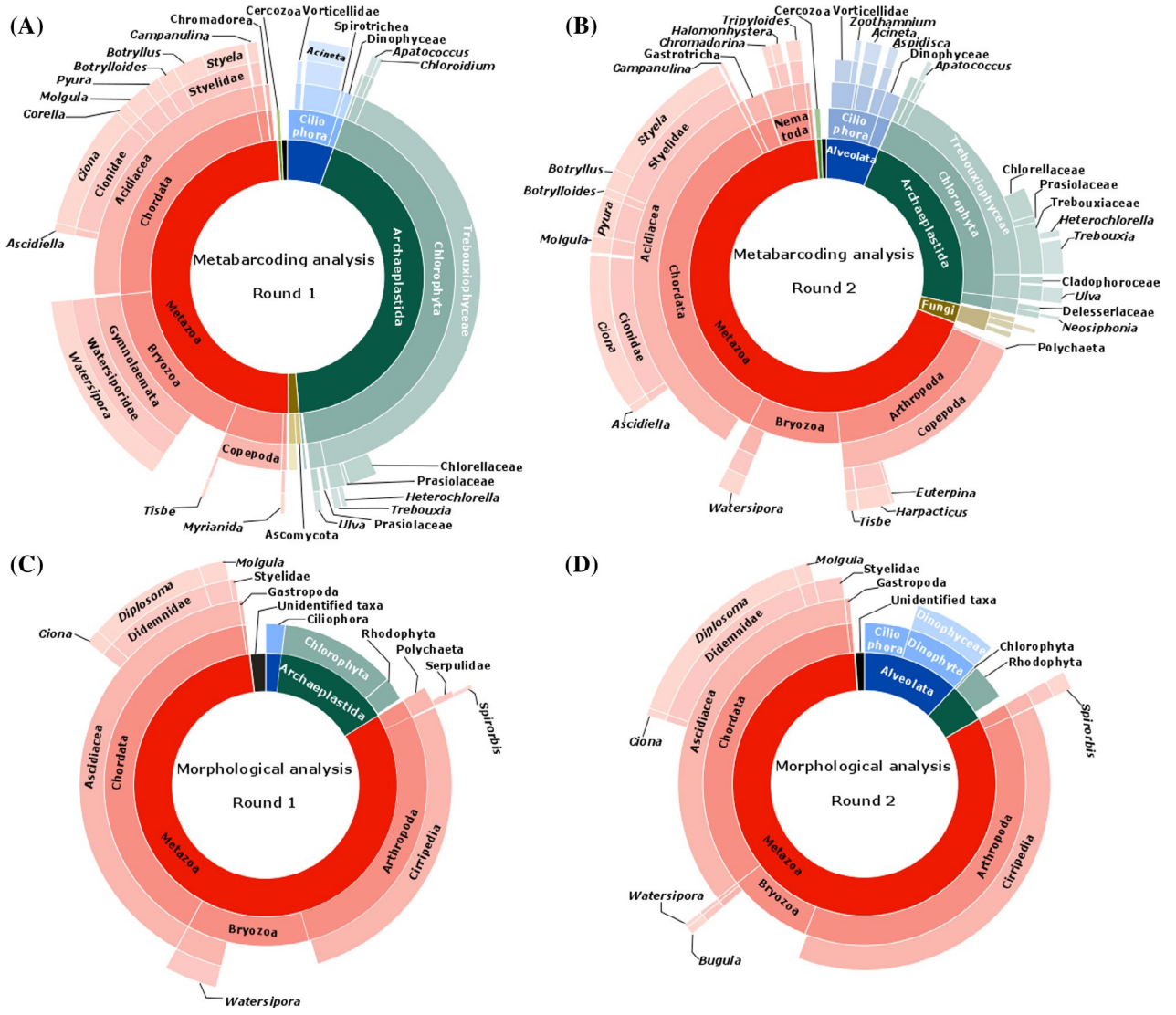

Figure 2. Partitioning of eukaryotic biodiversity detected on the settlement plates over two experimental rounds, based on the percentage of sequence reads for metabarcoding data (A and B) and the percentage of recruit abundance for morphology-based data (C and D).

The observed patterns in biofouling communities were largely driven by a few abundant taxa, contributing > 10% of sequence reads in one or more samples (19 in Round 1, and 20 in Round 2). For example, Chlorellales increased significantly in Round 1 over the exposure treatments, whereas multicellular animal taxa (particularly copepods and ascidians) contributed most in Round 2 (Figure 5).

Detection of early life stages of non-indigenous taxa

Overall, metabarcoding revealed the presence of 21 genera containing species that are non-indigenous or cryptogenic to New Zealand (Table S2). Although some of these genera contain both native and non-indigenous species (eg the ascidians *Molgula* and *Pyura*), some are entirely invasive, including *Ciona*, *Crassostrea* and *Watersipora*. Most of these were present on Day 1 plates in both Round 1 and Round 2 (17 and 19 genera correspondingly). Their contribution to assemblage structure was affected by exposure, experimental round and their interaction (non-parametric ANOVA, p=0.02, p<0.01 and p<0.01, respectively). In Round 1, these taxa dominated the early settlement on the plates (Figure 6A), contributing up to 90% of sequence reads. In Round 2, their contribution was more persistent over the exposure treatment, but still rather significant – up to 50% of the overall sequences.

The morphological analysis allowed identification of five genera containing known NIS (Table S3). All of these except for *Diplosoma* were also reported from the metabarcoding results. Their contribution to the biofouling assemblages across treatments ranged similarly to that detected using metabarcoding. However, the average

Figure 3. Partitioning of the taxa assigned from the internal control samples: PCR products of the amplified V4 region of the 18S rRNA gene of *Sabella spallanzanii*, *Ciona savignyi* and *Perna perna* mixed at equimolar (Control 1), 10:5:1 (Control 2) and 1:5:10 (Control 3) concentrations.

percentage values were consistent between methods on Day 5 plates only (Figure 6B). For morphological data, round and exposure also had a significant effect on the percentage of genera containing NIS (non-parametric ANOVA, p=0.02 and p<0.01, respectively), However, their interaction had only marginal effect (p=0.07).

Discussion

A large proportion of marine nuisance species are sessile, hard-substratum dwelling organisms (Cranfield et al. 1998; Hewitt et al. 2009) that become part of biofouling communities in artificial or natural habitats. Consequently, most guidelines for port surveys and risk assessments (eg HELCOM 2013; Awad et al. 2014) recommend biofouling communities as particularly important targets for sampling. This study highlights how metabarcoding can be used to complement traditional surveillance approaches and ultimately increase chances of timely detection of a potential pest. However, there are some important limitations that need to be considered and addressed in future research efforts for improving the robustness of the method. The findings of this study also provide valuable insight into the patterns of biofouling formation on artificial substrata and allow some practical conclusions and recommendations for further applications of metabarcoding.

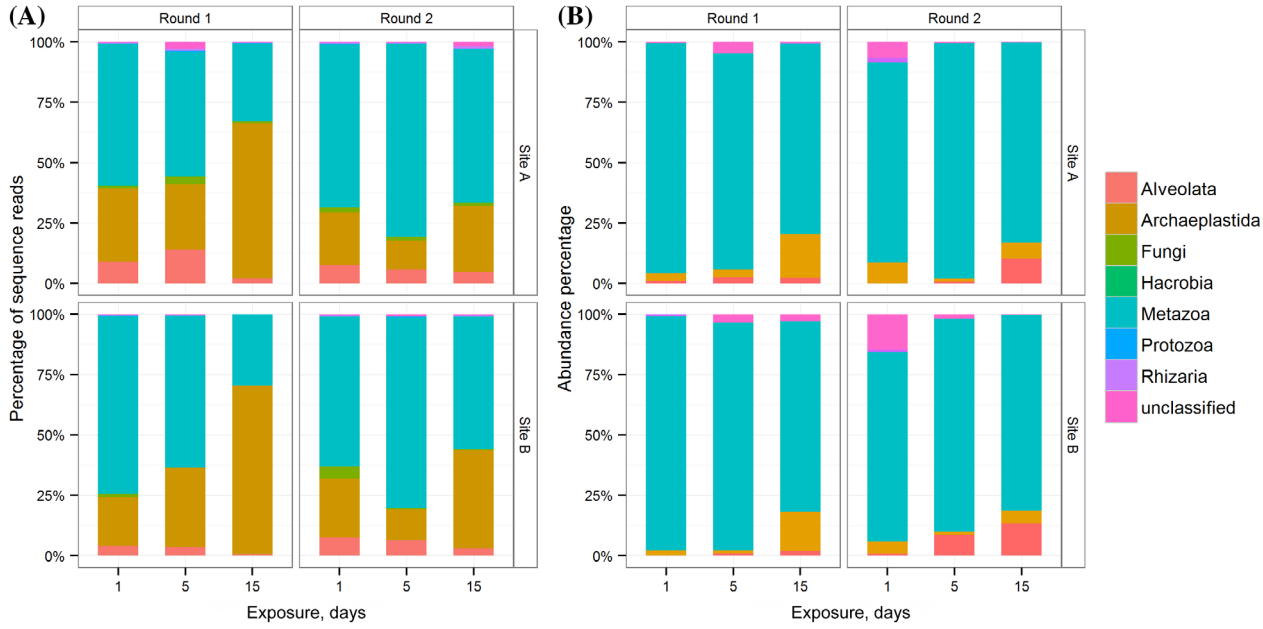

Figure 4. Shift in community composition (at the supergroup level) over three exposure treatments, based on metabarcoding (A), and morphological (B) data for each investigated site and round.

Detection of patterns in early biofouling communities

The present experiment considered three ecological factors: the temporal (experimental round) and spatial (experimental site) variability in recruitment patterns of different taxa, as well as time of substratum exposure to propagules in the water column. The findings support the results from a previous experimental study based exclusively on morphological analysis (Schimanski 2015), and evidenced that the most important factors influencing community composition were experimental round (timing of deployment) and exposure. The limited spatial variability in environmental conditions and a fairly homogeneous distribution of propagules, likely due to hydrodynamic peculiarities in the study area (Hart 2004), resulted in the early convergence of biofouling communities at the two experimental sites (Figure 4).

Many experimental studies have shown that the timing of introduction of new substrata can substantially influence the pattern and rate of succession in biofouling communities (eg Jenkins & Martins 2010). There is a distinct seasonality in recruitment patterns in temperate environments, with peak recruitment often occurring in summer (when this study took place). In February, many marine invertebrates spawn in New Zealand coastal waters (eg Tortell 1981; Gordon & Mawatari 1992; O'Driscoll et al. 2003), increasing the pool of propagules within plankton communities. This might explain the persistent prevalence of metazoan taxa during the second experimental round (Figures 4 and 5). Among metazoans, the molluscs, round and ringed worms tended to be the earliest settlers in both rounds, most likely benefiting from the lack of competitors or the facilitative effect of environmental cues from early biofilms (Zhao & Qian 2002).

Non-indigenous taxa might also benefit from reduced competition in early biofouling assemblages. According to Connell and Slatyer (1977), species that have evolved certain characteristics (eg, abundant, widely dispersing propagules, rapid growth and ability to withstand environmental stress) usually occur first on newly available substratum. Such characteristics correspond to the generally assumed features of successful NIS (Ricciardi & Rasmussen 1998; Piola & Johnston 2008). Therefore, it is not surprising that many metazoan genera detected in the early biofouling were those known to contain NIS or cryptogenic species. This was particularly evident in Round 1 (Figure 6A), before the massive recruitment by indigenous invertebrates occurred.

An important advantage of metabarcoding is the possibility of detecting potential marine pests at their earliest life stage. Additionally, by defining the patterns of their recruitment, knowledge of their ecological characteristics can be acquired and this may assist in improved risk assessments or management strategies. Visual identification and enumeration of recruits from early biofouling assemblages is complicated due to the cryptic morphology of microscopic organisms and larvae that are typically the most abundant first settlers on submerged substrata (Qian et al. 2007). The ability to identify non-indigenous taxa morphologically was limited due to their size and cryptic

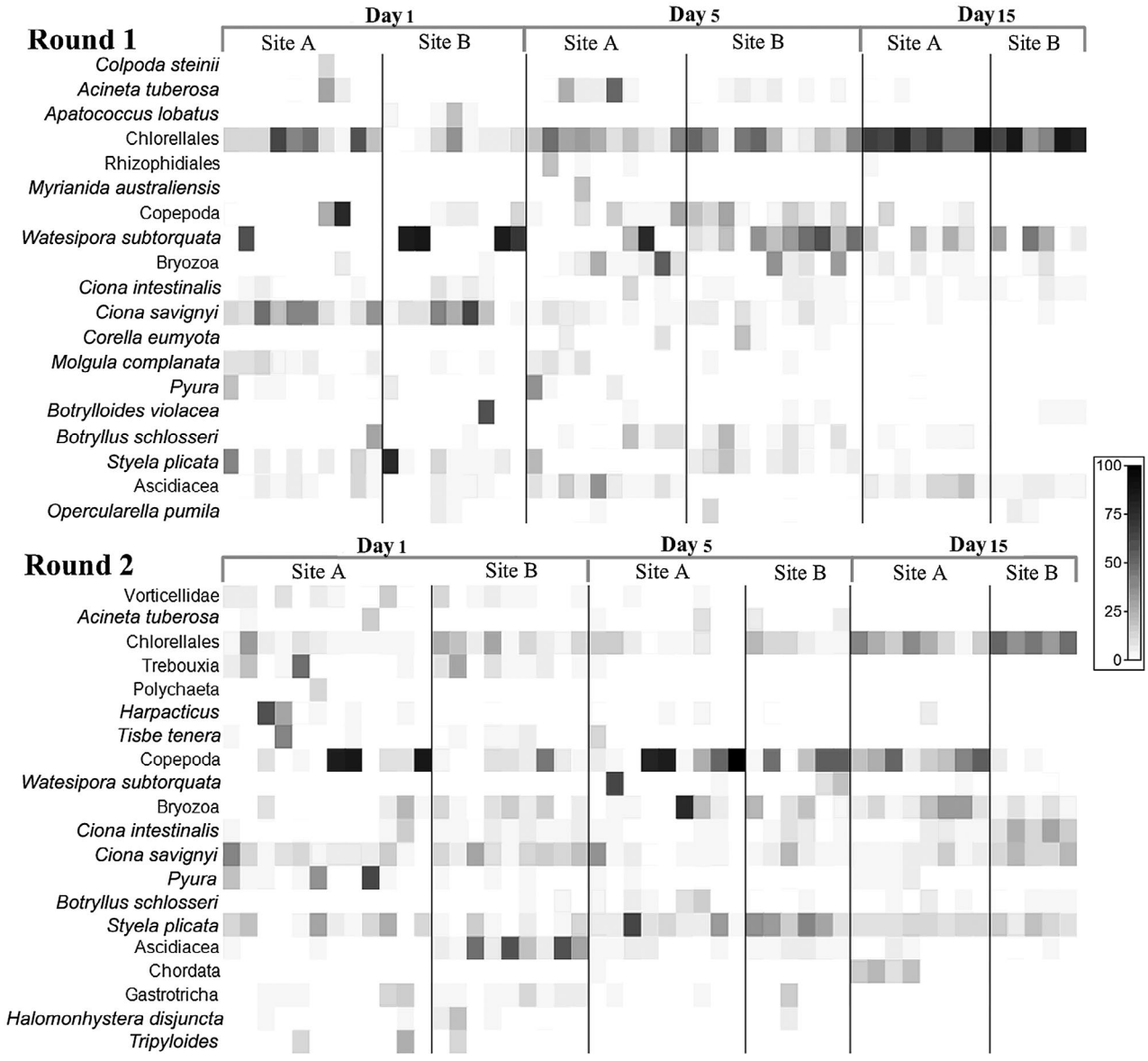

Figure 5. Shade plots representing the relative abundance of the most important taxa (contributing > 10% of the sequence reads in a sample) assigned at the lowest taxonomic level using metabarcoding. The white space denotes absence of the taxa; the depth of the gray scale is linearly proportional to the percentage of sequence reads. The site and exposure treatments are divided by vertical lines.

sub-adult stages on Day 1 plates and overgrowth by other species at the later succession phase (Day 15). Therefore the most accurate identification and quantification of NIS by morphology was on Day 5 plates, as was also demonstrated through a high consistency with metabarcoding results (Figure 6B).

Implications for NIS surveillance and pathway management

Among the dominant taxa derived from Day 1 plates, there were taxa that are considered biofouling pests in different global locations, including *Watersipora* (Ireland; Kelso & Wyse Jackson 2012), *Ciona* (Canada; Ramsay et al. 2007), *Molgula* (Germany; Buschbaum et al. 2012) and Botryllid ascidians (the Netherlands; Gittenberger 2007). Species from the same genera (eg *Watersipora subtorquata*, *Ciona intestinalis* and *Molgula manhattensis*) are non-indigenous marine organisms in New Zealand (Gordon & Mawatari 1992; Cranfield et al. 1998). The results from this study indicate that even a seemingly negligible level of biofouling may still harbor viable microscopic recruits and imply potential risks of NIS incursion. Therefore, for CRMS compliant vessels arriving from major transport hubs or locations with known pest infestations, additional molecular screening could be utilized to confirm low-risk status.

Many successful invaders are characterized by higher ecological plasticity and opportunistic adaptations

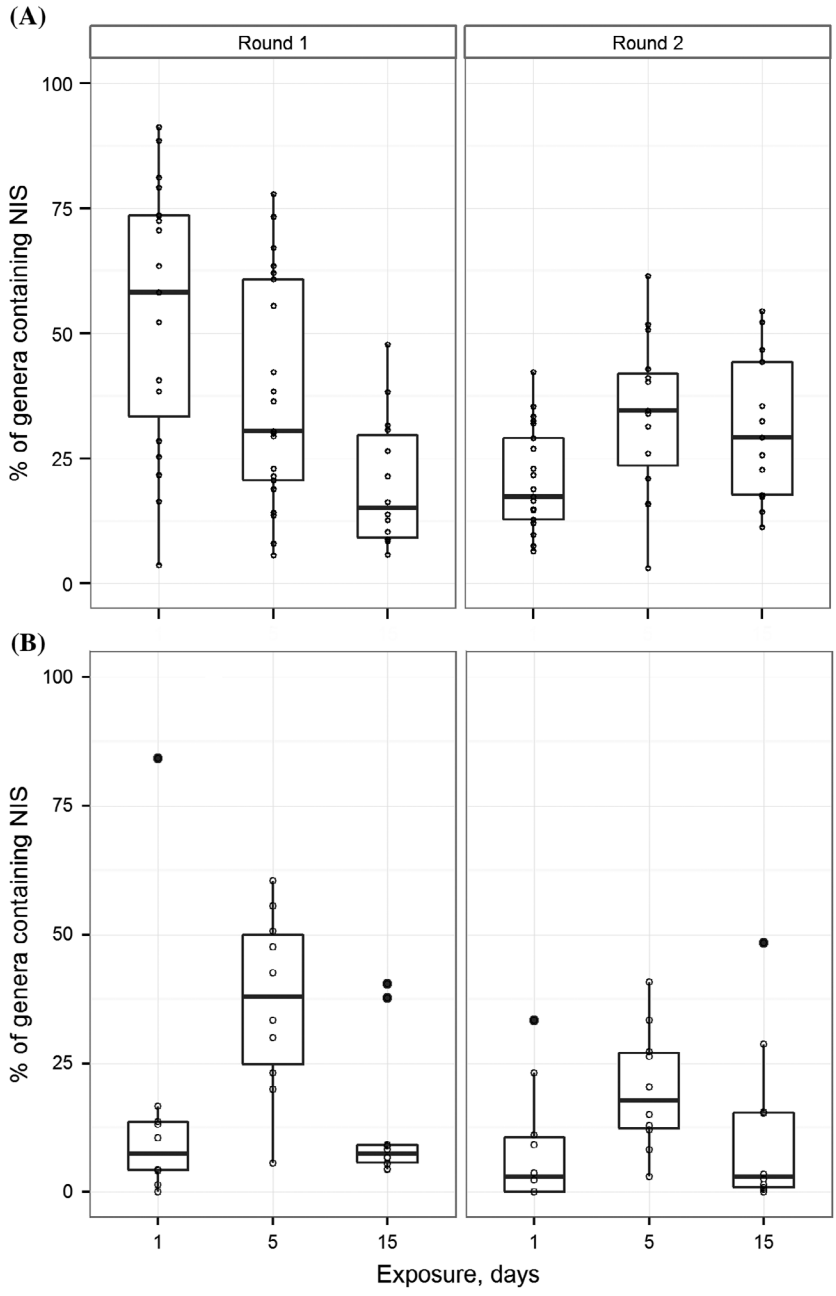

Figure 6. Relative abundance (percentage of sequence reads) of genera containing NIS species detected by metabarcoding (A) and morphological analysis (B) over the observation period, split by two experimental rounds. The black dots indicate outliers.

(Davidson et al. 2011), and demonstrate a faster response to resource availability. Therefore, first settlers taking advantage of novel substrata more effectively than other local sessile organisms are more likely to exhibit invasive behavior and could be prioritized in management agenda as candidate pest species. Metabarcoding can help identify these taxa from the scarce genetic material and may be useful as an early detection technique. Application of molecular screening of biological assemblages from biofouling may assist in the early identification of incursions on any type of submerged substrata, eg aquaculture facilities, port infrastructure, passive surveillance devices (eg settlement plates, and autonomous monitoring systems). Timely detection should indicate when preventive measures are needed to stop the aggregation of marine pests. This should allow focused management effort, targeting highest risk taxa and locations, thus increasing the overall cost-efficiency of marine pest management programs.

In general, metabarcoding is advisable as a complementary tool for port surveillance and could be easily combined with traditional approaches and passive sampling

strategies (eg settlement plates or panels). For efficient NIS detection, short-term deployments (one to five days) of sampling substrata in high-risk areas with several replicates over the summer season are advised. However, the amount and quality of DNA isolated from biofilms might be affected by short exposure (see Figure S1); therefore reasonable replication is highly recommended.

Considerations required before routine applications of metabarcoding

There are some practical issues that need to be carefully addressed before metabarcoding can be considered a proven off-the-shelf method for NIS surveillance. In this study, metabarcoding provided detailed information on the composition of eukaryotic assemblages over the studied period (Figure 2A and B) and at higher taxonomic levels demonstrated congruency with morphological identifications (Figure 4). The assignment of taxa at the lowest taxonomic levels (species or genera) need to be interpreted with caution as false positives can occur due to restricted specificity/resolution of markers and incompleteness of currently available reference sequence databases.

In this study, among the taxa identified unambiguously to genus level by morphological analysis, two (the bryozoan *Bugula* and the ascidian *Diplosoma*) were not discerned with metabarcoding. This is most likely due to the lack of corresponding reference sequences in the current version of the PR2 database. Under-representation of some taxonomic groups in databases may lead to biased results in HTS biodiversity assessments, and restrict the resolution and detection capacity at the taxonomy assignment step. The deficiency of taxonomic coverage in reference databases is one of the major current barriers to large-scale application of metabarcoding in biodiversity research (Ratnasingham & Hebert 2013; Dowle et al. 2015; Pochon, Zaiko et al. 2015). There is a need for country or regionally focused barcode reference library assembly to account for endemic biodiversity and possible regional variability in targeted barcode genes. Reference sequence databases for New Zealand marine taxa are lacking and this may result in imprecise taxonomic assignment (Dowle et al. 2015). In this study, only 60% of genera reported by metabarcoding were listed in the New Zealand Organisms Register (http://www.nzor.org.nz, Table S2). This could be due to either incorrect taxonomic assignments or incompleteness of the current marine biodiversity inventories.

Another limitation of metabarcoding is primer biases, which may favor the detection of certain taxa (Clarke et al. 2014). Some organisms (eg those that are rare, small or calcified) may remain unidentified using HTS due to poor preservation, different DNA extraction methods or PCR failure (Zhan et al. 2013; Dowle et al. 2015). This could explain why the polychaete worm *Spirorbis*, reported from 22% of the morphologically analyzed plates, was not identified in the HTS data, although represented by two reference sequences in the PR2 database.

Discrepancies may also arise from the high intraspecific variability of the barcode gene in some species. Although the V4 region targeted in the present study performed well both in terms of universality and sensitivity, it has limited resolution for species-level assignments across all groups. For example, the 18S rRNA gene region does not enable differentiation between the invasive South African brown mussel (*Perna perna*) and the New Zealand green-lipped mussel (*P. canaliculus*; Pochon et al. 2013). The *P. perna* DNA sample that was used in the present study as a positive control could only be assigned at the family (Mytilidae) level under the 97% similarity threshold (Figure 3), despite the presence of multiple (n=1934) representative sequences in the PR2 database. In contrast, ascidian species such as *Ciona intestinalis* and *C. savignyi* can be confidently differentiated using short barcodes such as the V4 region (Pochon, Zaiko et al. 2015). This highlights the need for future studies to investigate more than one gene in parallel and exploit recently available reference frameworks for multigene DNA barcoding (Chesters et al. 2015).

Given the short size of the targeted gene, the possibility that metabarcoding biofouling material also captures extracellular DNA signals or DNA traces from detritus cannot be excluded (Dell'Anno & Danovaro 2005; Pawlowski et al. 2014). However, this is less problematic if the method is applied for pest detection, as it will report the recent biodiversity present in fouling deposition or the wider environment. This might be considered an additional advantage over traditional surveillance approaches for NIS surveillance and early pest alert. If only living biodiversity is of interest (eg for pathway risk assessments), metabarcoding of environmental DNA and RNA can be conducted in parallel (Pawlowski et al. 2014; Pochon, Wood et al. 2015).

Despite these limitations, metabarcoding will assist in characterizing local marine biodiversity, potentially identifying cryptic species and highlighting groups for which better reference sequence coverage or taxonomic verification is required. This study has demonstrated that metabarcoding can provide high resolution taxonomic information, which may enhance the identification of biofilm recruits and enable detection of potential pests at the earliest stage of incursion. High-throughput sequencing enables the analysis of a large number of samples simultaneously. This is advantageous for upscaling survey efforts for early detection of populations without increasing the per-sample cost of taxonomic diagnosis.

Further refinements to the current methodology and improvements in reference sequence databases will facilitate the uptake of this technique for research and routine monitoring purposes.

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