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Metabarcoding as a tool to enhance marine surveillance of nonindigenous species in tropical harbors: A case study in Tahiti

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Abstract

Globalization has increased connectivity between countries enhancing the spread of marine nonindigenous species (NIS). The establishment of marine NIS shows substantial negative effects on the structure and functioning of the natural ecosystems by competing for habitats and resources. Ports are often hubs for the spread of NIS via commercial and recreational vessels. Prevention, detection, and mitigation efforts are required to avoid and manage the establishment of NIS in new ecosystems. In this study, metabarcoding approaches targeting the nuclear small-subunit ribosomal RNA (18S rRNA) gene and mitochondrial cytochrome c oxidase I (COI) gene were used to investigate planktonic and sessile (i.e., biofouling) communities and NIS at four locations in Tahiti, including two marinas and one port with varying anthropogenic impacts, and a relatively pristine site (Manava) used as a control. ASV richness values showed significant differences (18S rRNA gene: $p = .023$; COI: $p < .001$) between locations in the plankton samples, with the control site (low impact) having the highest diversity for both genes. ASV richness was also significantly different among locations for the biofouling samples in the COI dataset ($p = .002$). Community composition differed between locations with spatial patterns appearing stronger for the plankton samples compared with the biofouling samples. Detection of NIS based on selected lists of globally invasive species revealed a wide diversity of potentially invasive taxa especially in the more anthropogenically impacted regions. The use of a multigene approach improved the detection of NIS. This study demonstrates the utility of using a metabarcoding approach to routinely monitor areas most at risk from NIS establishment in Tahiti and other coastal nations. These coastal nations are vulnerable to shipping-mediated incursions, and baseline information is required for both native diversity and nonindigenous diversity.

KEYWORDS

biomonitoring, environmental DNA (eDNA), marine biosecurity, metabarcoding, nonindigenous species

1 | INTRODUCTION

Globalization has led to increased connections between countries, and this has played a key role in accelerating the spread of marine nonindigenous species (NIS) across many marine biomes (Bax et al., 2003; Seebens et al., 2013; Wonham & Carlton, 2005). Maritime exploration and trade have expanded substantially in recent decades, and thus shipping, including ballast water and biofouling, has become the main pathway for the transport of marine NIS (Molnar et al., 2008; Roche et al., 2015). As ship speeds have increased, the probability of NIS surviving translocations from remote locations is also enhanced (Carlton, 1989). In this context, ports and marinas act as hubs for incursions and a source for secondary spread of marine NIS (Molnar et al., 2008; Roche et al., 2015). Once established in a port, NIS can spread regionally via biofouling or water and associated debris entrained in bilge spaces of smaller recreational and commercial vessels (Acosta & Forrest, 2009; Fletcher et al., 2017; Johnson et al., 2001; McMahon, 2011; Mineur et al., 2008; Pochon et al., 2017), as well as via currents, marine debris, and other natural vectors (Carlton & Cohen, 2003). Some NIS have a high spreading capacity and cause adverse effects on native communities, and the structure and functioning of coastal ecosystems (Galil, 2007; Kotta et al., 2006; Wallentinus & Nyberg, 2007), sometimes leading to devastating effects on economies, coastal societies, and ecosystem services (Ricciardi et al., 2017; Wallentinus & Nyberg, 2007).

The cost of eradication is often high and success rates low once a NIS has become established (Crombie et al., 2008; Summerson et al., 2013). Prevention is, by far, the best approach to limiting the impact of NIS but it is often difficult to achieve (Bax et al., 2003; Crombie et al., 2008; Ojaveer et al., 2018; Summerson et al., 2013). Detection at the early stages of incursion is critical (Bax et al., 2003). Traditionally, surveillance programs have relied on techniques that use morphological identification of organisms (e.g., visual surveys by divers or identification of biofouling on settlement plates; David et al., 2019; Hewitt & Martin, 2001), which often miss cryptic or small organisms/life stages, are time consuming, expensive, and require taxonomic expertise (Abad et al., 2016; von Ammon et al., 2018a; Darling & Blum, 2007; Kim & Byrne, 2006).

In an effort to reduce the time and costs associated with monitoring, to improve standardization and sensitivity, and to provide earlier detection opportunities, recent developments in the field of molecular ecology have led to the rapid and incremental incorporation of these techniques into environmental surveys, including marine biosecurity surveillance (Bott et al., 2010; Chain et al., 2016; Comtet et al., 2015; Darling & Blum, 2007). The genetic material extracted in bulk from environmental samples (e.g., soil, sediment, air, and water) can be defined as environmental DNA (eDNA). This comprises not only DNA contained in the cells of an organism but also extracellular DNA originating from feces, urine, saliva, or dead cells (Taberlet et al., 2018). The analysis of eDNA can be effectively used to complement existing biomonitoring techniques through metabarcoding of entire biological communities (e.g., Carvalho et al., 2019; de Vargas et al., 2015; Fonseca et al., 2010; Keeley et al., 2018; Pearman et al., 2019; Shi et al., 2011). Environmental DNA metabarcoding has been proposed as a future tool for early and cost-effective screening for NIS (e.g., Brown et al., 2016; Chain et al., 2016; Chariton et al., 2010; Darling et al., 2017; Suarez-Menendez et al., 2020; Wood et al., 2013) from a range of environmental samples to complement marine surveillance programs. Extensive research efforts using metabarcoding and other species-specific real-time quantitative assays have focused on understanding detection limits, improving sampling and analytical methods, and elucidating the distribution and fate of nucleic acids from a range of NIS in controlled and field settings (von Ammon et al., 2018a, 2018b, 2019; Pochon et al., 2013, 2017; Rey et al., 2020; Wood et al., 2020; Wood, et al., 2019a, 2019b; Zaiko et al., 2016; Zhan et al., 2013). While these investigations have brought eDNA tools closer to routine implementation, molecular approaches are still largely limited in locations with minimum resources (Hewitt & Martin, 2001; Huhn et al., 2019; Nuñez & Pauchard, 2010; Ojaveer et al., 2018). There is now significant scope to adapt these approaches for use in administrative work plans seeking to establish marine biosecurity frameworks with restricted resources, lack of baseline biodiversity information or limited availability of taxonomic expertise. Incorporating metabarcoding-based techniques would assist regions particularly vulnerable to man-mediated biological invasions in protecting and managing their natural resources.

Here, we present a case study from French Polynesia, a world-renowned tourist destination, encompassing 118 islands distributed over 5.5 million km^2 which are divided into five archipelagoes. As in many other Pacific island nations, its unique biodiversity is currently at risk due to a significant increase in maritime (trade), commercial (aquaculture), and recreational activities, 90% of which are passing through Papeete harbor (www.portdepapeete.pf, accessed 08/04/2020). Currently, the main focus for biosecurity in French Polynesia is assessing introductions of NIS via touristic and commercial trading, but there is no specific marine pest management program in place. While efforts to DNA barcode all marine fauna are in progress in French Polynesia (http://biocode.swala.org/, accessed 14/04/2020), comprehensive baseline surveys of NIS throughout its territory are required. A recent report showed that 31 out of the 61 marine NIS identified across all French Overseas Departments are present in French Polynesia (UICN Comité français, 2019). Barcoding techniques have been utilized for the detection of mollusk NIS in French Polynesia (Ardura et al., 2015, 2016) with the presence of invasive mollusks being linked to boat traffic density (Ardura et al., 2015). However, there is an urgent need for additional comprehensive baseline surveys of NIS from ports and marinas in French Polynesia and the establishment of a long-term NIS surveillance program throughout the Pacific region. Scientifically validated biodiversity information from bioinvasion hot spots is also required by international legislation. Thus, for example, under Article 6 of the Ballast Water Management Convention (IMO, 2004), states are encouraged to undertake scientific research and monitoring to identify the exposure to NIS and to monitor changes over time (Abdulla et al., 2014).

The aim of this study was to conduct a pilot baseline survey applying eDNA metabarcoding protocols, targeting two genes (nuclear 18S rRNA gene and mitochondrial COI gene), to analyze plankton and biofouling samples collected from a relatively undisturbed coastal area (Manava), two marinas (Marina Taina and Marina Papeete) and the commercial port of Papeete. These four areas represent different levels of bioinvasion-related disturbance, that is, propagule pressure (Lockwood et al., 2005) inferred from proximity, and intensity of two major bioinvasion pathways: commercial vessels and recreational craft. We focus on characterizing the composition and structure of marine coastal communities and identifying putative NIS. We hypothesized that: (a) There will be differences in the richness and community structure and that fewer NIS would be detected in Manava, the relatively undisturbed coastal region, both in biofouling and plankton samples, and (b) the two different genes targeted in the current experiment will provide complementary information on the taxonomic composition of eukaryotes, thus increasing the potential for NIS detection.

2 | METHODS

2.1 | Field sampling

Field sampling was conducted between 14 and 15 November 2018 in four distinct locations (Table S1, Figures S1–S2) representing different disturbance levels in relation to shipping traffic. The control (i.e., proposed as undisturbed coastal area) site was the most Western sampling location at the Manava (M) hotel. From there, Marina Taina (MT) is located 2.7 km North and is characterized by an annual passage of ~450 recreational ships. A further 8.7 km North-East is Marina Papeete (MP), located adjacent to the Port of Papeete (PP). Marina Papeete was completely renovated in 2015 and has a total of approximately 1,250 boat passages a year, but is also likely to be affected by the main Port of Papeete activities. The Port of Papeete was first constructed in 1860 and is a hub for freight and interisland traffic around French Polynesia. This included ~ 900,000 tons of freight and 1.8 million passengers on interisland ferries (IEOM, 2019).

Two types of environmental samples (plankton and biofouling) were collected in triplicate at three sites for each location with the exception of Manava which only had a single site (Manava $n = 6$; other locations $n = 18$), giving a total of 60 samples (30 plankton and 30 biofouling samples; Table S1). Plankton samples were collected from each site (pontoon) using a plankton net (30 cm opening; 10-µm mesh size), to target larger phytoplankton (e.g., Diatoms, Dinoflagellates, etc) and smaller zooplankton, equipped with a weighted cod-end enabling vertical sampling through the water column (Figure S3a). The net was lowered to a maximum of 10 m depth and hauled—vertically to filter approximately 700 L. For shallower sites, the net was redeployed multiple times until approximately 700 L of water passed through (see Table S1). The concentrated plankton sample (ca. 500–700 ml) was then immediately filtered through one or two Whatman™ (3 µm, 47 mm dia.) filter(s) using a Millipore™ Sterifil™ filtration unit operated with a manual pump (Figure S3b). Filters were placed in sterilized DNAse-free tubes containing 1 ml of RNA-Shield™ buffer (Zymo Research, CA, California) and stored on ice. Between each collection site, the plankton net, and filtration unit were thoroughly rinsed with local seawater. Between each of the four sampling locations all sampling material was soaked in a solution of 5% hypochlorite and rinsed with tap water. Sterilized gloves were used at all times.

Three biofouling samples (ca. 2 cm^2) were collected at each site from haphazardly selected submerged surfaces (buoys, ropes, tires, pontoon structure) using stainless steel surgical blades (Table S1; Figure S3c and d). The sampling design allowed "soft" macro-organisms (e.g., algae) to be collected; however, it restricted the representative analysis of large hard-bodied organisms (e.g., mollusks). The samples were immediately placed in sterilized DNAse-free tubes containing 1 ml of RNA-Shield™ buffer (Zymo Research, CA, California) and stored on ice. Preserved samples were transported to the Cawthron Institute, New Zealand, for further processing.

2.2 | DNA extraction, polymerase chain reaction, and sequencing

Biofouling samples were centrifuged (15,000 *g*, 5 min) and the supernatant discarded. A single subsample of 0.25 g was taken from each sample and transferred into PowerBead Tubes of the DNeasy PowerSoil Kit (Qiagen, CA, USA). The plankton filters were directly transferred into PowerBead Tubes. All samples were homogenized via bead beating for 2 min using a 1,600 MiniG homogenizer (Spex SamplePrep, NJ, USA) and centrifuged (10,000 *g*, 1 min, 20°C; Eppendorf Centrifuge 5430R, Hamburg, Germany). Total DNA was extracted following the DNeasy PowerSoil protocol (Qiagen) on the QIAcube robot (Qiagen). A DNA extraction blank was included for every 24 samples (3 blanks in total).

Polymerase chain reactions (PCRs) were performed to amplify the V4 region of the nuclear 18S rRNA gene and a fragment of the mitochondrial COI gene. The primers used were (18S rRNA gene) Uni18SF: 5′-AGG GCA AKY CTG GTG CCA GC-3′ and Uni18SR: 5′-GRC GGT ATC TRA TCG YCT T-3′ (Zhan et al., 2013), (COI) mlCOIintF: 5′-GGW ACW GGW TGA ACW GTW TAY CCY CC-3′ and jgHCO2198: 5′-TAN ACY TCN GGR TGN CCR AAR AAY CA-3′ (Leray et al., 2013). Illumina™ overhang adaptors were attached to the primers to allow dual-indexing as described in Kozich et al. (2013). Amplifications were undertaken on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) in a total volume of 50 µl using 25 µl of MyFi™ Mix (Bioline, MA, USA), 1 µl of each primer, 20 µl of DNA-free water, and 3 µl of template DNA. Thermocycling conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 20 s, 52°C for 20 s, 72°C for 20 s, and a final extension of 72°C for 10 min. Negative (no template) controls were run alongside the samples in the PCR (1 control every 20 samples; three per marker in total). The 18S rRNA gene and COI amplification products were

cleaned and normalized using SequalPrep Normalization plates (Thermo Fisher Scientific) resulting in a concentration of ~ 1 ng/μl.

Samples (n = 67 for each gene) were prepared for sequencing on an Illumina Miseq™ platform at Auckland Genomics, University of Auckland, New Zealand. This was achieved following the Illumina 16S rRNA metagenomics library prep manual with the exception that after the indexing PCR, 5 μl of each indexed sample was pooled, and a single clean-up of pooled PCR products was undertaken instead of the samples being individually cleaned. Quality control was undertaken on a bioanalyzer before the library was diluted to 4 nM, denatured, and diluted to a final loading concentration of 7 ρM with a 15% PhiX spike. Raw sequence reads are deposited in the NCBI short read archive under the accession number PRJNA634820.

Bioinformatic pipelines for both 18S rRNA gene and COI sequences were identical unless otherwise stated. Cutadapt (Martin, 2011) was used to remove the primer sequences from the raw reads with a single mismatch being allowed. These trimmed sequences were subsequently processed using the DADA2 package (Callahan et al., 2016) within R (R Core Team, 2020). The reads were truncated to 230 and 228 bp (forward and reverse reads, respectively) and filtered with a maximum number of "expected errors" (maxEE) threshold of two (forward reads) and six (reverse reads). If reads did not meet this threshold, then they were discarded from further analysis. The first 10^8 bp were used to construct a parametric error matrix within DADA2, and following sequence dereplication sequence variants for the forward and reverse reads were determined. Paired-end reads were merged, after singletons were discarded, with a maximum mismatch of 1 bp and a minimum overlap of 10 bp. Chimeric sequences were removed within DADA2 using the consensus option in the removeBimeraDenovo script. For the COI dataset, anomalies in amino acid translations were detected using Multiple Alignment of Coding Sequences (MASCE; Ranwez et al., 2011). This program was used to translate and align the sequences against the MIDORI (Machida et al., 2017) reference database. This was undertaken in a two-step process. Firstly, query sequences were translated using the invertebrate translation code and aligned against a subset of the MIDORI database containing only invertebrates. Any sequences with a stop codon or possessing greater than two frameshifts were then translated using the vertebrate code and aligned against a vertebrate subset of the database. Any sequences containing a stop codon or possessing greater than two frameshifts were considered as pseudogenes and removed from further analysis.

Subsequent to chimera (and pseudogene) checking, the amplicon sequence variants (ASVs) for the 18S rRNA gene were taxonomically classified against the PR2 (Guillou et al., 2012) database using a two-step process. The DADA2 assignTaxonomy script, based on the rdp classifier (Wang et al., 2007), was run with a bootstrap cutoff of 0.9 and then repeated at a cutoff of 0.5 for classification of higher taxonomic ranks (family and above) which had not been previously classified.

For the COI dataset, taxonomic assignment was achieved using a combination of three approaches, as per Laroche et al. (2020) to reduce the number of unassigned sequences and increase taxonomic resolution: (a) the classification trees ("insect") classifier (version 5; Wilkinson et al., 2018) trained on the MIDORI database (Machida et al., 2017) and marine sequences from the GenBank nucleotide (nt) database (Benson et al., 2000); (b) using megablast from Blastn application (options: -evalue 0.001 -max_target_seqs 5 -task megablast -perc_identity 0.8; (Camacho et al., 2009)); and (c) Blastn (options: -evalue 0.001 -max_taget_seqs 5 -task blastn) on the entire GenBank nt database. For the Blastn methods, taxonomy returned from each hit (max 5 per query sequence) was assigned to the lowest common ancestor among hits. Parameters were based on analysis of the marine taxa in the MIDORI database as described in Laroche et al. (2020). To avoid overclassification, this assignment was then corrected based on a minimum percent identity value and minimum percentage cover (80%) for each taxonomic rank as in Laroche et al. (2020). Finally, results from the Insect classifier and the Blastn approaches were collated, and in the absence of conflicting results among methods, taxonomy was retained from the method with the highest resolution. In case of conflict, the lowest common ancestor among the different approaches was assigned.

To remove possible contamination, we used the maximum sequence count for each ASV as a removal threshold (Bell et al., 2019). This was done three times for the extraction, PCR, and sequencing controls. Thus, any ASV with fewer reads than the threshold was assumed to be from contamination and removed from further analysis. ASVs with reads higher than the threshold were reduced by the threshold number to take into account the contamination. We did not remove these ASVs completely as a possible source of contamination is among samples, and thus, complete removal would possibly remove genuine ASVs. Other methodologies to address possible contamination (e.g., max ASV count in the controls as a threshold) were investigated but found to limit the detection of rare NIS. As the detection of NIS was a major aim of this paper, the results from these more conservative methodologies were not assessed further.

The number of retained reads for each step of the bioinformatic pipeline is detailed in Table S2.

2.3 | Statistical analyses

To assess the community structure, relative proportional abundance was calculated for both the plankton net and biofouling samples on the unrarefied datasets for each region. For comparison between samples, each sample was rarefied to 7,600 and 17,200 reads for 18S rRNA and COI genes, respectively. This was a compromise between losing samples and reaching a plateau in the rarefactions curves and was independent of taxonomic classifications (Figure S4). Differences in the ASV richness (a combination of infraspecific and taxonomic diversity) of the communities were analyzed using one-way ANOVA for location and site separately (one factor: Location; 4 levels: Manava, Marina Taina, Marina Papeete, and Port of Papeete. one factor: Site; 10 levels). The ANOVA was undertaken on subsets of the dataset (plankton net and biofouling) for each gene separately.

The shapiro.test was used to test for normality. The data from the COI gene biofouling samples were log-transformed to meet normality assumptions. The leveneTest (package car; (Fox & Weisberg, 2019) was used to test for homogeneity of variances.

Multivariate analysis was undertaken on both datasets and sampling methods (plankton nets and biofouling) using the rarefied samples as described above. Principal coordinate analysis was undertaken to visualize the 2D representation of the ASV structure. Statistical differences were tested using the adonis function using Bray–Curtis distance matrices on the square root transformed data (one factor: Location; 4 levels: Manava, Marina Taina, Marina Papeete, and Port of Papeete) in the vegan package in R (Oksanen et al., 2007). Pairwise permutation tests were undertaken with RVAidememoire (Hervé, 2017). Linear regression analysis was undertaken assessing the similarity among samples and geographic linear distance. The geographic distance was calculated using the distHaversine function in the R package geosphere (Hijmans et al., 2017). Figures were constructed in R using the package ggplot (Wickham, 2016).

For the detection of putative NIS, a list of potentially invasive species was compiled based on available international databases of key threat species (von Ammon et al., 2018a) and species with invasion history elsewhere and not originating from the Pacific (AquaNIS, 2019). Taxonomic information for these species and the distribution was obtained from the WORMS database (Table S3; Chamberlain, 2020; Holstein, 2018; WoRMS, 2020).Comparisons were made between ASVs in either of the unrarefied 18S and COI datasets and those on the potential invasive species list.

3 | RESULTS

A total of 4,980 and 12,964 ASVs were detected with an average number of reads per ASV of 314 (sd = 5,261) and 336 (sd = 3,147) in the 18S rRNA and COI gene datasets, respectively. Of these, 3,425 (68.8%) and 498 (3.8%) ASVs could be assigned to the genus level while 1,564 (31.4%) and 461 (3.6%) ASVs could be assigned at the species level for the 18S rRNA and COI gene datasets, respectively. In the 18S rRNA gene data, the phylum Dinoflagellata had the highest number (418; ~27%) of the species-level identifications, while in the COI dataset, the phylum Mollusca accounted for ~ 50% (229 ASVs) of the species-level taxonomic classifications. Differences were shown in comparisons between the two gene datasets when ASVs were amalgamated at the different taxonomic levels of classification (Table S4). For instance, COI detected a wider range of classes within the phyla Annelida (1 class using 18S compared to 8 classified using COI), Arthropoda (18S: 8; COI: 15), and Ochrophyta (18S: 1; COI: 11), while protists such as Cercozoa (18S: 10; COI: 1) and Ciliophora (18S: 5; COI: 0) had a wider range of classes in the 18S data. There were also discrepancies in the percentage of total ASVs which could be assigned at the class level. Hexanauplia (Arthropoda, 18S: 18.5%; COI: 1.5%), Dinophyceae (Dinoflagellata, 18S: 18%; COI: 0%), Adenophorea (Nematoda, 18S: 5.9%; COI: 0%), and Polychaeta (Annelida, 18S: 5.2%; COI: <1%) had a higher number of ASVs in the 18S data. In contrast, Bacillariophyceae (Ochrophyta, 18S < 1%; COI: 10.7%), Insect (Arthropoda 18S: <1%; COI: 6.2%), Gastropoda (Mollusca 18S: 1.2%; COI: 4.7%), and Oomycetes (Heterokontophyta 18S: 0%; COI: 9%) had higher diversity in the COI dataset.

Across all locations, Arthropoda were dominant (relative average abundance per region: COI range: 35%–56%; 18S rRNA gene range: 64%–79%) in the plankton community for both the 18S rRNA and COI genes (Figure 1). The biofouling samples were more variable in the composition at the phylum level with Arthropoda contributing to a lesser extent, especially in Marina Taina (18S: 4%; COI: 22%). Ascomycota were substantial contributors in both gene sets in Port of Papeete (18S: 27%; COI: 34%), and Porifera were substantial contributors in Marina Papeete (18S: 22%; COI: 49%), respectively (Figure 1).

In both rarefied datasets, the plankton net samples had a higher average richness (18S: 231 ASVs; COI: 420 ASVs) than the biofouling samples (18S: 86 ASVs; COI: 241 ASVs). This was reflected at the phyla level as well with the plankton samples having more ASVs in Arthropoda and Annelida in both datasets (Table S5). Bacillariophyta and Mollusca in the COI dataset and Dinoflagellata in the 18S dataset also had higher ASV richness in the plankton. In contrast, the 18S dataset had more Nematoda in the biofouling subset. Significant differences (one-way ANOVA) were observed in the ASV richness between locations for both the 18S ($F = 3.776$; $p = .023$) and COI ($F = 7.27$; $p = .001$) in the planktonic samples (Figure 2). Pairwise comparisons indicated Manava, the control site, had a higher plankton diversity compared with Marina Papeete in both the 18S rRNA and COI gene datasets ($p < .001$), and Marina Taina ($p = .045$) and Port of Papeete ($p < .001$) had lower diversity than Manava in the COI alone. Patterns among locations for the richness of biofouling samples with COI showed a statistical difference (ANOVA: $F = 6.97$; $p = .002$), with Marina Papeete having a lower diversity than either Marina Taina ($p < .001$) and Port of Papeete ($p < .001$) (Figure 2). There was no statistical difference (ANOVA: $F = 1.26$; $p = .315$) in the 18S biofouling dataset. For the plankton samples, there was a significant difference in the ASV richness among sites for both the 18S rRNA gene (ANOVA: $F = 10.31$; $p < .001$) and COI (ANOVA: $F = 14.25$; $p < .001$) (Figure S5). Pairwise comparisons showed that there was a similar ($p > .05$) ASV richness at the sites in Marina Papeete for both the 18S rRNA gene and COI and at Marina Taina in the COI dataset. In both datasets in the Port of Papeete, PP3 had a significantly lower ASV richness compared to both PP1 and PP2. In the biofouling samples, there was a significant difference in the COI data (ANOVA: $F = 7.899$; $p < .001$) although most pairwise comparisons were not significant with the exception of MP2 which generally had a lower ASV richness than the other sites. For the 18S rRNA gene, there was no significant difference in ASV richness among sites (ANOVA: $F = 0.705$; $p = .683$) (Figure S5).

Statistical differences using PERMANOVA in the community composition among locations measured by Bray–Curtis were highly significant for both genes and sampling methods (Biofouling samples (18S: $F = 1.45$; $p < .001$, COI: $F = 2.33$; $p < .001$); Planktonic samples (18S: $F = 2.76$; $p < .001$. COI: $F = 2.42$; $p < .001$); Figure 3). Pairwise

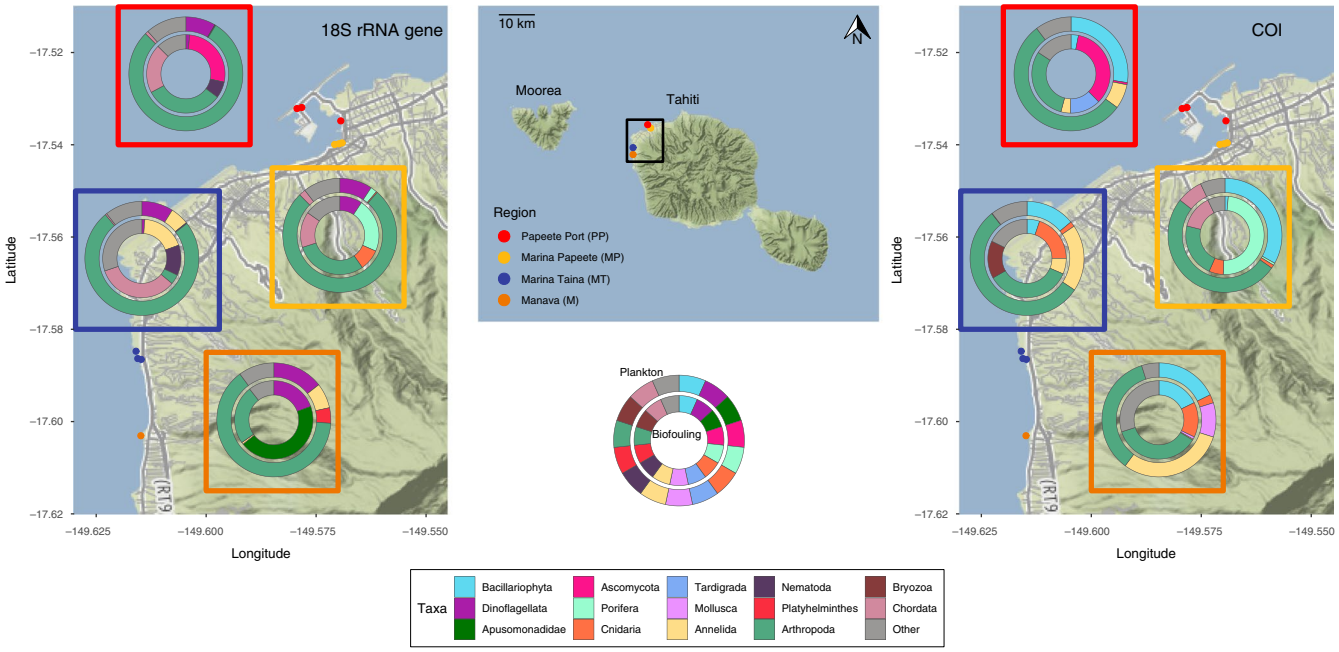

FIGURE 1 Composition (averaged across replicates and sites) of the communities at the level of phylum, for the biofouling (inner ring) and plankton (outer ring) for each location (Port of Papeete (PP), Marina Papeete (MP), Marina Taina (MT), Manava (M)) based on the 18S rRNA gene and mitochondrial cytochrome c oxidase I (COI) genes. See Figure S1 for details on sites within each marina and port locations [Colour figure can be viewed at wileyonlinelibrary.com]

comparisons indicated that all locations were significantly different for both sample types in the COI dataset. However for the 18S pairwise comparisons suggested that for the biofouling samples Manava is not significantly different from the other locations. The other locations are significantly different from each other. There were also significant differences in the community structure among sites ($p < .001$) for both genes and sampling methods (net versus biofouling). Regression analysis suggested that for all genes and sampling methods, there was a significant negative relationship between similarity (1-Bray–Curtis dissimilarity) with distance but this was stronger for the net samples ($R^2 = 0.243$ and 0.541 for 18S rRNA gene and COI, respectively; $p < .001$) than for the biofouling samples ($R^2 = 0.153$ and 0.115; $p = .018$ and 0.0228 for 18S rRNA gene and COI, respectively).

The detection of putative NIS was undertaken on both the 18S rRNA gene and COI unrarefied datasets. In total, 41 ASVs in the 18S rRNA gene (out of the 1564 ASVs classified at species level) and 9 in the COI (out of the 462 ASVs classified at species level) datasets were identified as belonging to invasive taxa with potential non-Pacific origins. These ASVs were from 14 genera encompassing both benthic/biofouling and planktonic taxa.

At most sites for the 18S rRNA gene dataset, less than 3 putative NIS were detected in both the biofouling and plankton samples (Figure 4a and b). However, a maximum of 7 NIS were detected in the plankton samples of PP2 with 6 observed in the plankton of MT2. In the biofouling samples, *Botryllus schlosseri* was detected 4 times, although the nonindigenous taxon *Bougainvilla* was observed to have the highest relative abundance (Figure 4a and b). In the plankton samples, *Botryllus schlosseri* was similarly the most frequently detected NIS acrossall sites (exceptPP3) with *Calocalanus plumulosus* and *Clytia noliformis* also found in more than 50% of sites. Overall *Botryllus schlosseri* also had the highest relative abundance in the plankton samples (Figure 4a and b).

For the COI dataset, there were only 4 putative NIS (Figure 4c and d) detected in the metabarcoding analysis. In both the biofouling and plankton samples, *Watersipora subtorquata* was the NIS most frequently detected in the COI data only being absent from M, MT3, PP1, and PP3. It also had the highest relative abundance of NIS in the dataset reaching ~ 21% at the Marina Taina (site MT2). Two other NIS (*Amathia verticillata* and *Bugula neritina*) were detected in the plankton samples but were only observed in one (PP1) and two (MP1 and MP3) sites, respectively. *Perophora viridis was detected in a single biofouling sample (MP3).*

4 | DISCUSSION

When establishing a marine biosecurity program, it is necessary to consider many aspects in relation to surveys and monitoring, such as concordance with the national strategies and international legislation (ICES, 2012; IMO, 2004, 2007), dominant vectors/pathways and ecosystems and economies at risk (Inglis, 2001). A crucial prerequisite is baseline information on native and nonindigenous biodiversity, against which future changes can be related to (Lehtiniemi et al., 2015). For example, to ensure successful implementation of the Ballast Water Management Convention, it is recommended to perform Port Biological Baseline Surveys in and around commercial ports to provide inventories of marine life and determine the presence, abundance and distribution of marine NIS in particular (Abdulla et al., 2014).

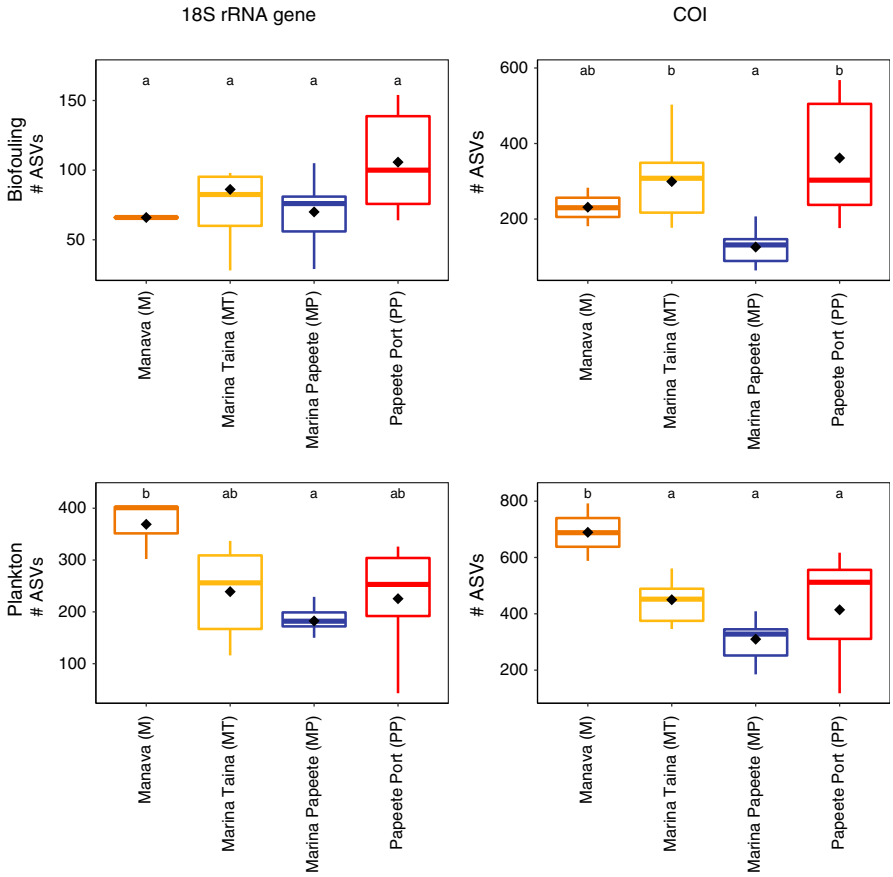

FIGURE 2 Amplicon sequence variant (ASV) richness for biofoulings and plankton samples using the 18S rRNA gene and mitochondrial cytochrome c oxidase I (COI) genes in each location (Manava (M), Marina Taina (MT), Marina Papeete (MP), Port of Papeete (PP)). The median is shown by the solid line across the box while the mean is indicated by the black diamond. Note difference in y-axis scales. Lowercase letters at the top of the plots indicate significant differences based on ANOVA tests ($p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

Baseline data enable risk-based priorities for long-term monitoring to be identified (Lehtiniemi et al., 2015). It is also important to maintain a sensible trade-off between time, cost, and spatial extent of the monitoring, especially when resources and sampling capacities are limited. Metabarcoding approaches are particularly appealing, since they can provide an unprecedented amount of biodiversity information at a significantly lower price per data unit and faster turn-around times (Glenn, 2011; Thomsen et al., 2012). Metabarcoding allows comprehensive inventories of marine biodiversity over extended spatiotemporal scales to be developed relatively rapidly (Rey et al., 2020). Before defining monitoring priorities and sampling design, in areas with diverse and often high levels of endemism, it is useful to identify metabarcoding-derived biodiversity patterns at different habitats, as well as the ability of different genes for discriminating putative NIS. In this study, COI and 18S rRNA gene metabarcoding screening of biofouling and plankton samples from high biosecurity risk sites (like ports and marinas) and a less disturbed area in Tahiti was undertaken to contribute to building-up a baseline of information and assist in designing a long-term biosecurity management program.

4.1 | Spatial patterns in the biofouling and planktonic communities

The sites chosen for this study had a variation in boat traffic, increasing from Manava to Marina Taina to Marina Papeete and the Port of Papeete, and were likely also affected by other anthropogenically derived stressors (e.g., oil and gas spills, vessels, and wastewater discharges), although these were not specifically considered in this study. All these factors may play a role in shaping marine biodiversity, and affecting differences in the patterns of ASV richness. In the present study, diversity patterns were not conserved between markers or sample type. In the Port of Papeete (the location with the highest marine traffic), ASV richness was significantly higher compared to Marina Papeete in the COI dataset and not significantly different in the 18S rRNA gene dataset. The reasonably pristine Manava area was characterized by higher ASV richness in the plankton samples, but not in biofouling. Alpha diversity metrics may not necessarily be directly linked to disturbance gradients, as similar values can be obtained with completely different species complexes (Hewitt et al., 2005, 2010). Therefore, multivariate analyses are commonly considered in addition to basic biodiversity metrics to differentiate spatial patterns in community composition and structure and relate them to disturbance levels (Chariton et al., 2015; Hewitt et al., 2005; Pearman et al., 2018). We observed among-location differences in community structure. Overall, there was a negative relationship between community similarity and distances. This could reflect the natural variability in marine communities with closer communities generally more similar to those further away due to dispersal limitations (Fonseca et al., 2014; Leray & Knowlton, 2016; Pearman, et al., 2018). However, there was also likely an anthropogenic impact gradient which correlates with the distance gradient meaning that

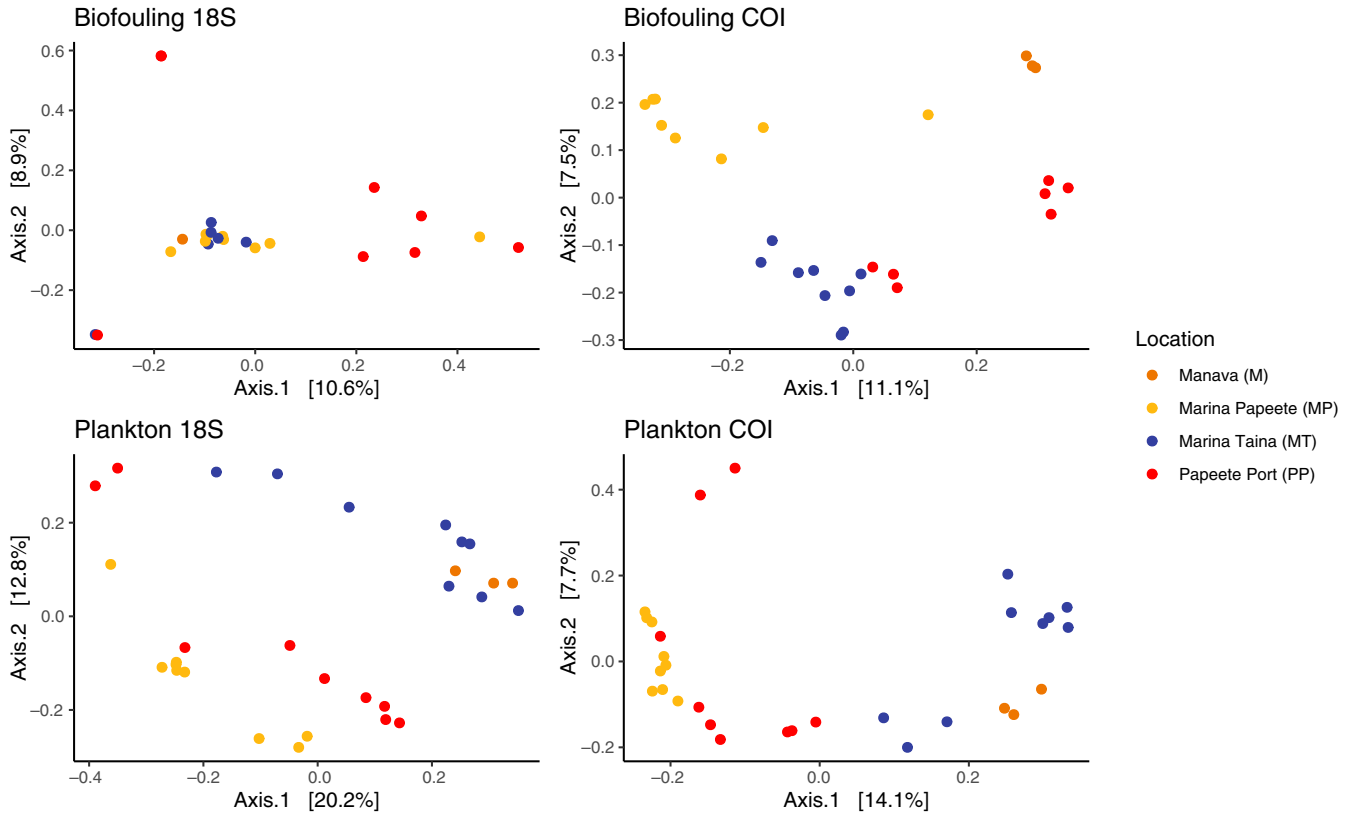

FIGURE 3 Principal coordinate analysis plots based on Bray–Curtis dissimilarities (log-transformed) of the biofoulings and plankton communities using data from the 18S rRNA gene and mitochondrial cytochrome c oxidase I (COI) genes. Points are colored per location [Colour figure can be viewed at wileyonlinelibrary.com]

the differences in the community could also be down to disturbance effects. These results may set the basis for a larger spatial analysis of metabarcoding results including more disturbed and pristine locations across French Polynesia. While assessing the levels of anthropogenic disturbance (e.g., polycyclic aromatic hydrocarbon levels, fecal coliform levels) at each site was beyond the capabilities of the current study, inclusion of environmental variables should be considered in future studies. This could then increase the knowledge of the effect of anthropogenic stressors on marine planktonic and benthic communities and the potential drivers of change. Temporal sampling of these stations could then be undertaken to better understand seasonal variations in community composition with Rey et al. (2020) having shown that seasonal variations can affect the metabarcoding community retrieved in fouling environments. This would enable more robust advice on sampling design for maximized biodiversity recovery with eDNA and higher probabilities of detecting rare and small taxa, for example, propagules of newly introduced NIS (Rey et al., 2020).

In this study, putative NIS in both planktonic and biofouling samples were found. We constructed a list of globally known invasive species whose native distribution did not include the south Pacific. While this list is unlikely to be exhaustive, it does highlight the existing state of NIS infestation and provide a baseline for further monitoring surveys using metabarcoding and traditional approaches in this region. Across the two gene datasets, a total of 50 ASVs, corresponding to putative NIS, were observed. These ASVs covered 14 genera and belonged to a combination of benthic/biofouling (8 genera) and planktonic (6 genera) taxa across 8 phyla. Shipping has previously been indicated as a anthropogenic pathway for the spread of these taxa to new regions (e.g., Coutts & Dodgshun, 2007; Gollasch et al., 2009; Mackie et al., 2006). Overall, our result showed that Manava, the site with the least intensity of operating bioinvasion pathways/vectors, had low levels of putative NIS both in terms of richness and relative abundance, with the exception of one planktonic sample for the 18S rRNA where the Cnidarian genera *Clytia* contributed substantially.

The successful colonization of NIS is a combination of colonization pressure (the number of NIS being introduced) and the propagule pressure (the number of individuals introduced and the number of introduction events) to the location (Lockwood et al., 2009). International ports, such as the Port of Papeete, are expected to display relatively high colonization and propagule pressures due to their international nature (Leclerc et al., 2018), and this conclusion was supported in the 18S rRNA gene dataset, as most NIS were detected in the Port of Papeete. However, Marina Taina had a similar number of putative NIS detected and the relative abundance of NIS in the two marinas studied was often higher than in the port. There are a few possible reasons for these observations. Firstly, artificial substrates, which are ubiquitous in marinas, provide ample space for colonization of NIS (Leclerc et al., 2018). Indeed, NIS are

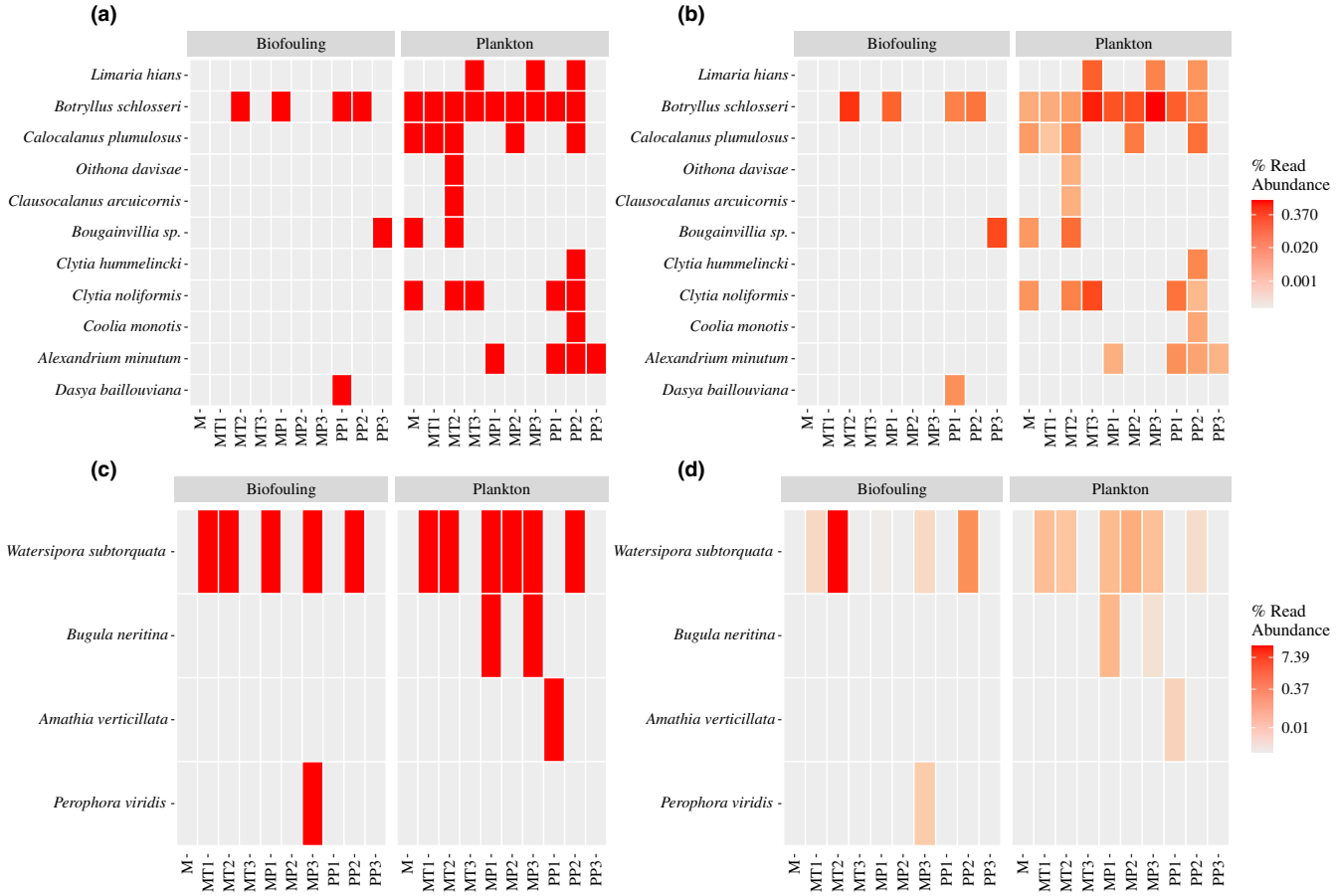

FIGURE 4 Presence/absence heatmaps for potentially nonindigenous species found in either the biofouling or plankton using; (a) 18S rRNA gene or (c) mitochondrial cytochrome c oxidase I (COI) gene in the different locations. Red = present. Relative abundance (%) of reads assigned to potentially nonindigenous species found in either the biofouling or plankton using; (b) 18S rRNA gene, or (d) mitochondrial cytochrome c oxidase I (COI) gene in the different locations (M = Manava; MT = Marina Taina; MP = Marina Papeete; PP = Port of Papeete). Details on specific sites within marina/port locations are shown in Figure S1 [Colour figure can be viewed at wileyonlinelibrary.com]

often highly opportunistic, and compared to native species are more likely to occur on artificial rather than adjacent natural substrates (Connell, 2001; Dafforn et al., 2012; Foster et al., 2016). Secondly, marinas tend to be semi-enclosed environments compared to more open harbors and this results in higher water residency and thus—concentration of NIS propagules (Floerl & Inglis, 2003). These factors increase the propagule pressure in marinas and may also affect concentrations of their eDNA in water, enhancing their detectability with metabarcoding approaches. Thirdly, while ports may act as a primary hub for introductions, secondary spread via recreational boating to nearby marinas is thought to play an important role in regional dispersal of marine NIS (Lacoursière-Roussel et al., 2012). The dense network of boating routes, slow speeds undertaken by recreational boats, and lack of control of their biofouling and bilge water discharges would increase the chances of spread around and between marinas (Foster et al., 2016; Lacoursière-Roussel et al., 2012; Pochon et al., 2017).

Previous barcoding work on molluscan and barnacle NIS in French Polynesia has been undertaken in Moorea (Ardura et al., 2015, 2016), but none of these species were detected in the current dataset although this is likely to be due to the geographical separation of the studies although some species did not have representatives in the databases used in the current study. In general, the relative abundance of putative NIS in our datasets was < 1.5% of total reads. The proportion of reads cannot reliably be used as a proxy for absolute abundance or biomass (Fonseca, 2018). However, it does give an indication that NIS do not comprise a significant proportion in the studied communities. The possible exception is Marina Taina (site MT2), where 20% of the COI reads in the biofouling were accounted for by a notorious invasive bryozoan, *Watersipora subtorquata*, which could indicate substantial shifts in community composition and functioning (Gallardo et al., 2016). This species is native to the Caribbean–Atlantic region (Mackie et al., 2006), but with extensive invasion spread as far as Australia and New Zealand (Mackie et al., 2012). It is able to rapidly colonize surfaces of degraded antifouling paints and thus quickly spread across ship hulls, acting as the main transport vectors (Mackie et al., 2006). Interestingly, eDNA signals for this species were predominantly found in samples from the port and marinas, especially in plankton samples. With the caveat that sampling effort was limited in the current study, no sequences of *W. subtorquata* were found in either the plankton or biofouling samples in the control site Manava, suggesting that its dispersal

has not yet progressed far beyond the impacted hub sites. The species has a relatively short larval dispersal time (1–2 days) (Mackie et al., 2006), and therefore, a secondary spread would be reliant on actively operating anthropogenic pathways (i.e., shipping) in the region. It is advisable to explore other sites in Tahiti (North-West Coast and elsewhere) to confirm the current distribution range of *W. subtorquata* and assess the risks of further invasion spread.

In the 18S rRNA gene dataset, *Botryllus schlosseri* was the most frequently observed NIS being found in all locations. This species is a native of Europe (Ben-Shlomo et al., 2010), but was supposedly transported into other regions via ballast waters or hull fouling (Dijkstra et al., 2007). *Botryllus schlosseri* is a colonial ascidian, and these organisms have been indicated to produce allelopathic chemical compounds that inhibit the recruitment of other species onto various substrates (Dijkstra et al., 2007). An ASV belonging to *B. schlosseri* was found in the planktonic dataset at Manava. The colonization of this location with *B. schlosseri* could have an impact on the surrounding community composition and having potential implications for aquaculture activities. The majority of the other ASVs observed in Manava belonged to Cnidarians (*Clytia* and *Bougainvilla*). Predatory hydroids can consume fish larvae and copepods in large quantities and can thus have an impact on fisheries when populations of these Cnidarians reach high numbers (Madin et al., 1996). Furthermore, top down control on copepods could wipe out native species and allow for invasive copepods, such as *Oithonia davisae*, to become established in communities (Altukhov et al., 2014).

In the present study, a higher diversity of putative NIS was measured within the plankton community and it has been previously shown that obligatory benthic species can be detected with higher probabilities from water samples than biofouling (von Ammon et al., 2019; Wood, et al., 2019a). Indeed, in the current study the majority of NIS ASVs belonging to sessile organisms (Genera: *Botryllus*, *Limaria*, *Perophora*, and *Dasya*) were found more frequently in the planktonic samples than the biofouling samples. The exception was *Dasya* and *Perophora* which were found in a single biofouling sample. This result could be due to the relatively different sampling effort that can be undertaken using either methodology. Biofouling samples are often limited by the amount of material that can ultimately be extracted using commercial DNA extraction kits (in this case 0.25 g). Using filters for the plankton samples, negates, this issue since large volumes of water can easily be filtered. This would increase the chances of detecting rare or low abundance species compared with small biofouling sample sizes. Another potential methodological issue for consideration is the propensity of distinct biofouling organisms to successfully settle on and colonize different materials and surfaces (von Ammon, et al., 2018b). A variety of materials would likely be required to detect a full range of biofouling organisms.

Planktonic metabarcoding surveys could be used for rapid community screening over large areas. These results could then inform sampling designs for more targeted biofouling sampling which should preferably encompass potential reservoirs of NIS (possibly identified through the plankton sampling) as well as a range of abiotic conditions and areas such as active ship berths (Hewitt & Martin, 2001). This more targeted biofouling approach could possibly be undertaken using species-specific methodologies (e.g., quantitative or droplet digital PCR), which is more efficient when searching for a particular species (Wood, et al., 2019a; Zaiko et al., 2018). This would provide a more cost-effective approach to detecting NIS especially as eDNA extraction protocols are often limited by the sample volumes that they use, preventing large biofouling biomass to be rapidly extracted.

DNA can be detected without the species being present in the system as short fragments of DNA can persist in the environment (Deiner et al., 2017). This can lead to misinterpretation of the distribution of species based on just metabarcoding surveys and caution in the reporting of NIS just based on metabarcoding detection is urged (Darling, 2019). Metabarcoding, however, can aid in determining areas of particular risk from NIS and help in developing focused management strategies. Areas with high detection rates of putative NIS or the presence of species of particular concern (e.g., species known for their adverse effects elsewhere) could be subjected to the more labor intensive morpho-taxonomy approaches such as diving surveys to confirm the actual location and abundance of source populations (Darling, 2019).

4.2 | Advantages of the multimarker approach for biosecurity applications

The selection of markers and primers sets is an essential consideration when undertaking surveys of ecosystems using eDNA methodologies and becomes critical when deriving richness estimates and detecting the presence of NIS in the marine biosecurity context (Duarte et al., 2020; Grey et al., 2018). The lack of detection or misidentification of NIS can have costly management or trade implications and may even be exposed to legal challenges (Darling & Mahon, 2011; Darling et al., 2020). Environmental DNA methods are certainly not immune to false-negative and false-positive results (Cristescu & Hebert, 2018). One approach to improve the detection of rare NIS is to increase the number of sample replicates (both extraction and PCR). While this approach was not undertaken in the current study, increased replication has been shown to improve diversity measurements (Ficetola et al., 2015; Lanzén et al., 2017), and future metabarcoding studies aimed at detecting rare NIS should carefully consider the levels of replication to improve confidence in limiting false negatives of NIS. Among the many factors that may contribute to misidentification of marine NIS (e.g., eDNA sampling and isolation methods; (Rey et al., 2020), marker choice and primer artifacts are believed to contribute most (Kelly et al., 2017).

Leray and Knowlton (2015) have previously suggested that a combination of the 18S rRNA gene, which provides a broad overview of the eukaryotic domain and has an extensive reference database including marine species, and a hypervariable marker such as COI for high taxonomic resolution, provides a complementary analysis of organisms within communities. Many studies have adopted

this approach and corroborated its benefits (Drummond et al., 2015; Harasewych et al., 1997; Ogedengbe et al., 2011; Zhan et al., 2014). For marine NIS detection, however, the caveat is that the 18S rRNA typically lacks phylogenetic resolution at the species level and the COI may cause taxonomic bias due to the lack of conserved primer-binding sites along this highly variable region (Clarke et al., 2017; Deagle et al., 2014; Tang et al., 2012). This is also evident in the current dataset where, for example, *W. subtorquata* was classified to the species level using COI but this resolution was not achieved in the 18S rRNA gene dataset. This would have important implications in management decision as this invasive species would not have been detected if the 18S rRNA gene had been used in isolation. The present study also showed differential diversity results between the two markers for several classes. For example, Bacillariophyceae, Insecta, Gastropoda, and Oomycetes had a higher proportion of richness in the COI data compared to the 18S dataset, while Hexanauplia, Dinophyceae, and Adenophorea showed the inverse pattern. These differences in taxonomic classifications may be due, in part, to differences in the amplification efficiency of primers for different taxa (Leray & Knowlton, 2015). These known artifacts may impact results, which can have important implications in the marine biosecurity context (von Ammon et al., 2018a; Darling et al., 2018, 2020; Wood, et al., 2019a).

While differences were observed in the taxonomic classifications obtained from both primers, broadly similar trends were detected across regions for both ASV richness and structure although these tended to be stronger with the COI dataset. It is likely that due to the higher phylogenetic resolution (often at subspecies level), the COI reflects small-scale biogeographic differences between sampling sites while the 18S rRNA is more conserved and thus may not be able to resolve species-level differences in the composition. However, to screen for the presence of NIS in the marine environment, the use of a multigene/multitrophic approach is more advisable as it provides complementarity in taxonomic profiles (Pearman, et al., 2018), and is also increasingly being touted for marine biodiversity surveys (Jeunen et al., 2019; Ortega et al., 2020; West et al., 2020).

Bioinformatic pipelines are an important consideration when using molecular methodologies. In this experiment, we utilized amplicon sequence variants (ASVs) which represent a combination of infraspecific and taxonomic diversity and have a finer resolution than previously used operational taxonomic units which clustered reads at specific similarity thresholds. While the use of ASVs is likely to increase richness estimations (due to the inclusion of infraspecific variation) and taxonomic redundancy, it reduces the possibility of artificially clustering closely related species. In NIS studies, the clustering of related species could have an impact with NIS being incorrectly grouped with non-NIS relatives, leading to potential misidentifications, an issue that has previously been reported for metazoans in Canadian ports (Brown et al., 2016). A further important limitation of metabarcoding for biodiversity surveys is the incompleteness of reference databases used for taxonomic classifications (Carugati et al., 2015; McGee et al., 2019; Wangensteen et al., 2018). The lack of representative sequences in a database can lead to the misclassification or nonclassification of ASVs. Indeed in the current data, only 3.6% of the COI ASVs could be assigned to a species level. In terms of NIS detection, this can have important implications and thus increased efforts have to be made for consolidating current (e.g., 18S rRNA and COI) sequence databases of key threat NIS and closely related species, as well as for exploring new taxonomically informative markers (von Ammon et al., 2018a; Jamy et al., 2020; Stat et al., 2017; Weigand et al., 2019). Thus, collaborations between morphological taxonomists and molecular ecologists are required to improve reference databases especially for poorly characterized taxa and/or key threat NIS (Darling et al., 2017). This is especially important in regions that have received comparatively less attention (McGee et al., 2019).

5 | CONCLUSION

This study contributes valuable information which will assist in the development of metabarcoding-based port biological baseline surveys and biosecurity monitoring programs around French Polynesia and other Pacific regions. The derived biodiversity data will enable the establishment of inventories of aquatic organisms inhabiting ports and adjacent areas and provide biological data against which future changes in aquatic communities, including NIS, can be measured. Even with relatively small sampling effort over a confined geographical area, our data allowed the identification of sites with increased likelihood of NIS detection (marinas), substantial spatial differences in marine biodiversity and a few putative NIS with high invasive potential (*W. subtorquata*, *B. schlosseri*). These data can be revisited as taxonomic reference databases improve and regional species inventories can be retrospectively updated, thus refining the biological baselines. The multigene approach used in this study enabled the detection of a greater number of putative NIS based on a list of global invasive species. Based on the results presented here, we advocate for the use of multigene metabarcoding approaches targeting planktonic communities to be used as a rapid assessment over large spatial areas, followed by metabarcoding of biofouling assemblages and morpho-taxonomic approaches to confirm the presence of NIS. This two-step approach would allow for initial baseline screenings to be conducted at larger spatial scales, followed by more targeted follow-up studies. Further research is required to improve knowledge on the seasonal and interannual patterns in these communities and inform on the frequency of sampling required for accurate detection of NIS.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTION

XP, MZ, and SP designed the experiments and undertook the sampling. UA did the laboratory work, while JKP and OL undertook the bioinformatics. JKP, UA, and XP wrote the paper, while SAW, AZ, OL, MZ, and SP contributed to drafts of the paper.

DATA AVAILABILITY STATEMENT

Raw sequence reads are deposited in the NCBI short read archive under the accession number: PRJNA634820.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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