Declaration

Reference Number: MEEF2019008

Project Title: Habitat conservation by high resolution mapping of population connectivity: oyster reef recruitment patterns in the Pearl River Delta

Project Leader: Dr Bayden D. Russell

I hereby irrevocably declare to the MEEF Management Committee and the Steering Committee of the relevant Funds including the Top-up Fund, that all the dataset and information included in the completion report has been properly referenced, and necessary authorisation has been obtained in respect of information owned by third parties.

Signature:_____  ___

Project Leader, Dr Bayden Russell

Date:_____11 February 2022____

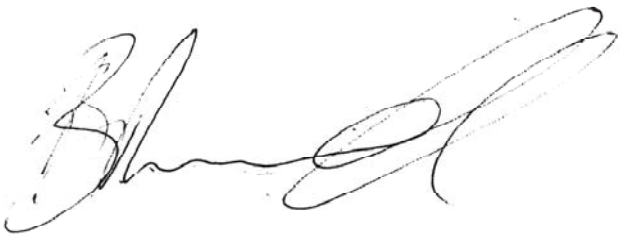
5.9.4.3. The project leader or the representative authorised by the Recipient Organisation must also sign in the statement of account attached to the completion report, or sign in the completion report, the following declaration, warranty and undertaking:-

"I hereby irrevocably declare, warrant and undertake to the MEEF Management Committee and the Steering Committee of the relevant Funds including the Top-up Fund, that I myself, and the Organisation:-

1. *do not deal with, and are not in any way associated with, any country or organisation or activity which is or may potentially be relevant to, or targeted by, sanctions administered by the United Nations Security Council, the European Union, Her Majesty's Treasury-United Kingdom, the United States Department of the Treasury's Office of Foreign Assets Control, or the Hong Kong Monetary Authority, or any sanctions law applicable;*

2. *have not used any money obtained from the Marine Ecology Enhancement Fund or the related Top-up Fund (and any derived surplus), in any unlawful manner, whether involving bribery, money-laundering, terrorism or infringement of any international or local law; and*

3. *have used the funds received (and any derived surplus) solely for the studies or projects which further the MEEF Objectives and have not distributed any portion of such funds (including any derived surplus) to members of the Recipient Organisation or the public."*



Final Report

Habitat conservation by high resolution mapping of population connectivity: oyster reef recruitment patterns in the Pearl River Delta

(MEEF2019008)

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Disclaimer

Any opinions, findings, conclusions, or recommendations expressed in this report do not necessarily reflect the views of the Marine Ecology Enhancement Fund or the Trustee.

Part 1: Executive summary

This project surveyed oyster habitats in five locations across the western coastlines of Hong Kong to identify the species diversity of oysters, the genetic diversity of the dominant oyster species. In addition, the microbiome associated with marine habitats, in particular bacterial communities, drive many of their functions (e.g., nutrient cycling), so taxonomic diversity of bacterial communities inhabiting oyster reefs in Hong Kong was also sampled to provide a first indication of the potential for microbiome-mediated functioning in these habitats. Finally, environmental DNA was tested as a method to identify the diversity of eukaryotes inhabiting oyster reefs.

Contrary to predictions based on the published scientific literature, we found that *Magallana angulata* and *M. sikamea* were the two dominant oyster species in natural oyster habitats in western Hong Kong, not *M. hongkongensis* as previously reported. In line with the greater species diversity than expected, the oyster populations that were sampled were also genetically diverse, meaning that they should be more resilient to environmental conditions. We also found that microhabitat structure in oyster reefs affects the bacterial diversity in the benthos, whereby greater bacterial diversity is associated with habitats with more structural complexity. While the functions associated with this greater diversity were not quantified, this finding provides the first indication that habitats with greater diversity of the habitat-forming species (i.e. oysters) and structural complexity may provide greater function and services.

Therefore, based on these findings, we recommend that not only should future oyster restoration efforts aim at increasing the population sizes of oysters in Hong Kong, but

care should also be taken to ensure oyster reefs are restored in a way that resembles their historically natural structure and diversity.

Part 2: Project title and brief introduction of the project

Title

Habitat conservation by high resolution mapping of population connectivity: oyster reef recruitment patterns in the Pearl River Delta

Description

Oyster reefs play a central role in marine ecosystem functioning. They influence nutrient cycling, filter the water column, and provide habitat for a variety of micro- and macro-organisms, therefore driving the ecosystem by enhancing water quality and boosting complexity in the food-web structure. They provide a rich feeding ground for a variety of organisms, including many commercially and recreationally important species. Unfortunately, in Hong Kong, oysters have been heavily harvested for a millennium and the oyster reefs are now functionally absent, leaving only small, degraded oyster habitats in very few locations. As such, there is currently a major push to restore these reefs, and the functions that they provide, to Hong Kong's waters, yet these efforts will not guarantee a sustainable and functional oyster reef unless we have a mechanistic understanding on the main constituents of a healthy oyster reef.

Biodiversity refers to not only the diversity of species (as commonly thought) but also genetic diversity within species and habitat diversity within systems (Glowka et al., 1994). Each level of biodiversity contributes to the stability of an ecosystem in its own unique way and hence should not be overlooked. This project aims at studying biodiversity in oyster reefs using a variety of approaches and seeks to derive a set of guiding principles for future oyster reef conservation programs.

Work schedule and completed activities

Below is a table outlining the original planned activities and work schedule. Against each is status of completion.

Activity (including Planning, Recruitment)	Date	Time	Venue	Status and comments

Recruitment of a research assistant	July 2019	1 month	HKU	**Completed**
Planning field trips	July 2019	1 month	HKU	**Completed**
Field trips (survey and sampling)	April 2020 – May 2020	2 months	PRD, Lantau Island, west Hong Kong	**Completed**. As noted in the project extension form, the border restrictions because of the COVID-19 pandemic (which are still in force) meant that sampling was not possible at the original sites planned in Mainland China. Therefore, sampling effort was increased in Hong Kong to increase the scientific inference in the population genetics of the oysters in Hong Kong. (see Methods and Figure 1, below, for detail).
Laboratory sample preparation and analysis	May 2020 – June 2020	2 months	HKU	**Completed**
Data interpretation	July 2020 – August 2020	2 months	HKU	**Completed**
Reporting and preparation of final report	September 2020-December 2020	3 months	HKU	**Completed**

Assessment of Project Effectiveness

Original (pre-pandemic) project goals

The original project proposal had three objectives (as stated in the original proposal):

1. Identify and characterize the reef building oyster populations (*Crassostrea* spp.) along the PRD, northern Lantau Island and western Hong Kong, and survey the oyster density and size distribution in each population.

2. Identify the source populations for oysters in the PRD, mapping the connectivity between oyster populations and quantifying the genetic diversity by using a population genetics approach
3. Develop reference maps of population connectivity by using GIS and directional networks.

There were, however, substantial issues with achieving these original project goals due to the restrictions imposed under the COVID-19 pandemic. Therefore, a revised set of project goals were approved in the project extension/revision application – these are noted below and effectiveness evaluated against the revised goals.

Effectiveness of project measured against original project goals:

1. **Identify and characterize the reef building oyster populations (*Crassostrea* spp.) along the PRD, northern Lantau Island and western Hong Kong, and survey the oyster density and size distribution in each population.**

We successfully surveyed and characterised the oyster populations in the western waters of Hong Kong that were identified/planned in the project proposal. However, border closures which were implemented at the start of the COVID-19 pandemic (and are still in place) meant that we were unable to sample any of the planned populations on the mainland side of the border.

2. **Identify the source populations for oysters in the PRD, mapping the connectivity between oyster populations and quantifying the genetic diversity by using a population genetics approach**

We successfully mapped the genetic linkages and genomic structure of the surveyed oyster populations of the western waters of Hong Kong (see revised objective 2 below). However, as with objective 1 above, the border closures made it impossible to sample the oyster populations in mainland China, so it was impossible to identify linkages between the China and Hong Kong oyster populations.

3. **Develop reference maps of population connectivity by using GIS and directional networks.**

To develop maps of population connectivity it is necessary to sample populations over a larger spatial scale than was possible with the border closures – for this original objective to be achievable the oyster populations would need to be sampled. Therefore, while we have developed the necessary information for the Hong Kong populations, further population genotyping of the Chinese oyster populations would be necessary to fully achieve this original goal.

Revised project goals

Below we report on the effectiveness of the project against the revised goals presented in the approved extension of project, in which a the updated workplan was outlined. This updated workplan and objectives were based on the travel restrictions put in place and border closure with Mainland China because of the COVID-19 pandemic, which were still in force following the completion of the project.

Objective 1: Identify the species diversity of oysters in Hong Kong

It is broadly believed that the majority of oysters found in the western waters of Hong Kong are *Magallana hongkongensis* (formerly *Crassostrea hongkongensis*). It is likely that this belief stems from the species name of the oyster (M. *hongkongensis*) and that it is a native oyster which has been used in the oyster aquaculture industry for over 700 years.
Our data (Part 3, below) clearly demonstrate that this commonly held belief is incorrect, but rather that *M. hongkongensis* is one of the least abundant oysters in natural habitat.

The goal of identifying the species diversity of oysters which make up the natural remnant oyster habitats in Hong Kong was successfully completed (Part 3, below).

Objective 2: Population genotyping and genetic diversity of oysters

The most common oyster species which make up oyster habitats in Hong Kong are *M. angulata* and 32 *M. sikamea*. Therefore, these two species were chosen as the target for Objective 2, to identify the genetic diversity of oysters making up natural remnant habitats. The complete mitogenome of *M. angulata* and *M. sikamea* were successfully assembled. Our data show that there are no barriers between genetic mixing in these oyster populations but there could be some strong selective pressures on the populations.

The data on the genetic diversity of oysters in Hong Kong were published in a collaborative paper with The Chinese University of Hong Kong:

Xie Y, Huang EYY, Nong W, Law STS, Yu Y, **Cheung K**, Li Y, Wong CF, Yip HY, Joyce P, Chan KM, Chu KH, **Russell BD***, Falkenberg LJ*, Hui JHL* (2022) Population genomics, transcriptional response to heat shock, and gut microbiota of Hong Kong oyster *Magallana hongkongensis*. *Journal of Marine Science and Engineering*, **10**, 237.

The MEEF funding was acknowledged in this paper.

The goal of documenting the genetic diversity of oysters which make up the natural remnant oyster habitats in Hong Kong was successfully completed (Part 4, below).

Objective 3: Biodiversity associated with oyster habitats?

One of the overarching tenents of oyster reef restoration is that the structural complexity and density of the habitat increases biodiversity of associated organisms. Whether the density of the oyster habitats has any effect on biodiversity of associated organisms is, however, rarely assessed. We effectively used environmental DNA (eDNA) as an efficient and cost-effective method for assessing this relationship. We demonstrate that this technique is effective, but it is most useful for identifying the diversity and turnover of smaller organisms (especially microorganisms) associated with different densities of oyster habitats, likely because the more mobile species can move between habitat patches. This finding does, however, support the use of eDNA over multiple scales to identify biodiversity of associated organisms.

The goal of documenting the biodiversity of organisms associated oyster habitats in Hong Kong, and demonstrating the use of eDNA as proof-of-concept, was successfully completed (Part 5, below).

Part 3: Species diversity of oysters in HK

Introduction

Without the ability to identify the components of the ecosystem (in this case, species) properly, we cannot make any meaningful claims about the mechanisms governing the normal functioning of the ecosystem, let alone predicting the response of the system to changing environments. Lam & Morton (2004) conducted an oyster survey on the eastern waters of Hong Kong and reported twelve species of oysters. However, to our knowledge, similar surveys have not been performed on the western side of Hong Kong. Thus, for the first part of this project, we present the first large scale effort in characterizing the species identity of oysters found in naturally occurring oyster habitats in the western waters of Hong Kong.

Shell morphology of oysters is a trait with extremely high phenotypic plasticity; accurate morphological identification of oyster species is considered a challenging, if not impossible, task. Previous accounts suggest that the most abundant oyster species in the western waters of Hong Kong is *Magallana hongkongensis*, “Hong Kong’s oyster”. This species has an estuarine affinity and so is biologically suited to the western waters which are dominated by the outflows of the Pearl River. In addition, this species is widely accepted to be the species which is cultured in the commercial oyster aquaculture industry based in Deep Bay, with spat (juvenile oysters) sourced from the nearby Guandong oyster production areas and imported into Hong Kong for culture. However, there are several other *Magallana* species inhabit the region, but the relative abundance of these species is unknown. Therefore, to avoid misidentification based on plastic morphological feathers, we used DNA sequence data to obtain reliable taxonomical identity of the oyster species (e.g., Wang & Guo, 2008)in the surveys and provide the first accurate assessment of the relative abundance of oyster species in the western waters of Hong Kong.

Methods

Oysters were collected from each of the five sites (n = 36 individuals per site) on the west coasts of Hong Kong (Figure 1). Only three oysters were collected from Yi O since a recent, unexplained mortality event prior to the sampling meant that only three live oysters were found in the area on the sampling day. The five sites are Pak Nai (22°26'25.2"N 113°56'47.6"E), Tai Ho Wan (22°17'53.6"N 113°58'18.5"E), San Tau (22°17'17.0"N 113°55'35.4"E), Yi O (22°14'04.6"N 113°50'53.9"E) and Shui Hau (22°13'07.9"N 113°55'31.9"E).

To limit the scope of the study, an effort was made to identify the oysters by shell morphology, with the aim of only collecting oysters of the genus *Magallana*. Morphological identification was performed by referring to the descriptions made by Lam & Morton (2004). Upon collection, oysters were placed on ice and transported back to the laboratory. Oysters were shucked and adductor muscles were dissected, preserved with pure ethanol, and stored at -20°C until further analysis.

DNA was extracted from the adductor muscles using the Qiagen DNeasy Blood & Tissue Kit following manufacturer's instructions. DNA samples were subject to quality check with agarose gel electrophoresis (0.8%) and UV spectrometry (NanoDrop™ One, Thermo Scientific™), then stored in -20°C until further analysis. A section of the *Cox1* gene was amplified with the primer pair LCO1490/HCO2198 (Folmer et al., 1994); the PCR was set up using *Taq* PCR Master Mix Kit (Qiagen) following manufacturer's instructions. PCR conditions were as followed: 1 cycle of denaturation step at 95 °C for 3 min; 35 cycles of 45 s at 95 °C, 45 s at 51 °C and 1 min at 72 °C; and a final extension step at 72 °C for 10 min. PCR products were examined on agarose gel (1.5%) and submitted to BGI Hong Kong for Sanger Sequencing. Sequence results were blasted against the NCBI nucleotide collection database using the megablast algorithm to confirm the sequence identity. To test for independence between site and species composition, Fisher's Exact Test was performed with simulated p-value in R v 4.0.2 (R Core Team, 2020).

Figure 1. Oyster survey sites.

Results

Sequencing reaction was successful for 144 out of the 147 samples. Species identity of the 144 oysters sampled were as below; oyster species composition was different between different sites ($p < 0.001$, Fisher's exact test). *M. sikamea* was dominant in Pak Nai and *M. angulata* has widespread distribution in Lantau. Despite efforts were made on only collecting *Magallana spp.*, some *Saccostrea cucullata* were accidentally collected.

Table 1: Oyster species composition of surveyed oyster reefs in western Hong Kong. Data are the number of individuals in each species genetically identified from each species.

Species	Pak Nai	Shui Hau	San Tau	Tai Ho Wan	Yi O	Total
M. angulata	0	17	30	20	2	69
M. sikamea	35	18	2	0	0	55
M. hongkongensis	0	0	1	4	0	5
M. dianbaiensis	0	0	0	0	1	1
M. ariakensis	0	0	1	0	0	1
S. cucullata	0	1	1	11	0	13
Total	35	36	35	35	3	**144**

Discussion

Contrary to popular belief, *M. hongkongensis* was rare in the natural oyster habitats that were surveyed in the western waters of Hong Kong. The two most abundant *Magallana* spp. in western Hong Kong are *M. angulata* and *M. sikamea*; both species are documented to have wide-spread distribution along the South China coastline (Wang et al., 2010, 2013), but this is the first documentation of their abundance relative to other species in Hong Kong.

The results of the study shows that oyster reefs around Hong Kong have different oyster species composition which seems to be geographically separated. For example, the northern most site sampled, Pak Nai, was dominated by *M. sikamea* even though it is within the aquaculture zone which is generally assumed to be entirely comprised of *M. hongkongensis*. This finding alone casts some question on the species identity of the oysters which are being brought from Guandong to Hong Kong as spat for grow-out in the aquaculture industry. In contrast, Tai Ho Wan and San Tau on the north coast of Lantau Island are dominated by *M. angulata*, while the more oceanic influenced Shui Hau has an even proportion of *M. angulata* and *M. sikamea*.

Nonetheless, these results should be interpreted with caution. While there seems to be a geographic pattern to the species composition of oysters in the western waters, a much larger sampling effort would be required to conclusively link relative species abundances to any geographical or environmental gradients. Of note is that we only have data from one year. Oysters are well known to have extremely high fecundity, which can differ substantially under different environmental conditions and between species. Further, harvest pressure is extremely high in Hong Kong, with few individual oysters surviving beyond one year of age in the intertidal (Lau et al. 2020). Therefore, there is a possibility that the oyster species composition in oyster reefs could change from year to year depending on both harvest pressure and locality-specific reproduction within different species (Hedgecock et al., 2007; Hedgecock & Pudovkin, 2011; Sun & Hedgecock, 2017). To elucidate any such dynamic change in assemblages, sampling would need to occur over multiple seasons to identify both drivers and responses to oyster species diversity.

For future oyster reef restoration programs, care should be taken to ensure the appropriate oyster species are targeted or transplanted to the restoration site. Each of the species have different environmental tolerances, not only for settlement and growth, but for peak reproduction. Therefore, if restoration is to succeed, species which can survive, grow, and reproduce in any particular location must be targeted – not the species which is thought to be the most common based on historical work or “local knowledge”. In particular, shell morphology should not be used as the sole identification feature of the target species; in the study some *S. cucullata* were collected despite expert knowledge and the team specifically targeting only specimens of *Magallana* spp. This exemplifies the challenge in accurate taxonomical identification of oysters by examining shell morphology. We therefore advocate routine use of genetic markers when accurate taxonomical identification of oysters is needed.

Part 4: Genetic diversity of *M. angulata* and *M. sikamea* in HK

Introduction

Genetic diversity is essential to the health of an ecosystem. Not only does genetic diversity of a population contribute to its resistance to environmental disturbances (Hughes & Stachowicz, 2004; Nevo et al., 1986; Reed & Frankham, 2003), it also promotes the productivity of ecosystems (Aguirre & Marshall, 2012). Hence, there has been an increase in awareness of genetic considerations in conservation programs in the recent decade (Laikre et al., 2010). Yet, the incorporation of genetics considerations in conservation efforts requires baseline understanding on the demography and genetic landscape of the population(s) of conservation interest (Gaitán-Espitia & Hobday, 2020), and such information is currently lacking for oysters in Hong Kong. In the second part of this project, we present a study on the genetic diversity of the two most abundant oyster species in the western waters of Hong Kong, *Magallana ariakensis* and *M. sikamea*.

Methods

Following the first part of this project, DNA samples from *M. angulata* (n = 38) and *M. sikamea* (n = 32) (Table 2) were sent to Novogene (Tianjin, China) for shotgun sequencing. 350bp sequencing libraries were constructed, multiplexed, and sequenced on Illumina NovaSeq 6000 platform (2 x 150 bp).

Table 2. Number of specimens sent for shotgun sequencing for the two species of oysters

Collection site	*M. angulata*	*M. sikamea*
Pak Nai	-	18
San Tau	10	2
Shui Hau	16	12
Tai Ho Wan	10	-
Yi O	2	-

Mitogenomes were assembled with *GetOrganelle v 1.7.3.1* with the default animal mitogenome settings (Jin et al., 2020). The sequencing reads were mapped to the reference mitogenome of the respective species (RefSeq accession number NC_012648.1 and NC_012649.1) with K-mer sizes of 21, 45, 65, 85 and 105. The resulting assemblies were

imported to Bandage *v 0.8.1*, where they were visualized as de Bruijn graphs for manual inspections and exported to fasta format for downstream analysis. To validate the assembly, all assembled mitogenomes were blasted against the NCBI nucleotide database to check for similarity with the mitogenome of the respective species.

Assembled mitogenomes were aligned with *MAFFT v4.475* with *–globalpair* setting enabled (Katoh & Standley, 2013); statistical parameters were calculated in *DNAsp v 6.12.03* (Rozas et al., 2017); Tajima's D test and Fu's F test was performed to test for the neutrality and pairwise F_{ST} was calculated to detect for population structure (Fu, 1997; Tajlma, 1983; Wright, 1950). A minimum spanning haplotype network was drawn for the mitogenome with *PopART v 1.7* (Leigh & Bryant, 2015).

Results

The complete mitogenome of 38 *M. angulata* and 32 *M. sikamea* were successfully assembled. All the assemblies were blasted against the NCBI standard nucleotide database and returned good alignment against the mitogenome of their respective species, with 100% query coverage and > 99% sequence similarity.

A total of 34 and 32 haplotypes were observed among the 38 *M. angulata* and 32 *M. sikamea* sampled, respectively. Haplotype diversity was high for both species (Table 3); In contrast, nucleotide diversities were low. Tajima's D is negative in *M. sikamea*, suggesting overabundance of rare alleles. Fu's Fs value was negative for both species, suggesting the excess of rare haplotypes when compared to the predictions under neutrality. High abundance of low frequency alleles and haplotypes are often observed in populations experiencing selective sweeps or population expansion.

Table 3. Genetic diversity metrics for the two species of oysters. n = number of samples, k = number of haplotypes, PS = number of polymorphic sites, *H* = haplotype diversity, π = nucleotide diversity

Species	**n**	**k**	**PS**	***H***	***π***	**Tajima's D (significance)**	**Fu's Fs (significance)**
M. angulata	38	34	479	0.993	0.004	-1.576 ($p > 0.10$)	-2.584 ($p < 0.05$)
M. sikamea	32	32	925	1	0.005	-2.264 ($p < 0.01$)	-3.461 ($p < 0.02$)

Pairwise Fst showed little population structure between *M. sikamea* collected from the three sites (Table 4). On the other hand, moderate genetic difference was observed between *M. angulata* specimens collected from San Tau and specimens collected from

other locations. The absence of strong population structure was also reflected in the haplotype networks (Figure 2). There were no strong barriers to gene flow between the sampled populations from the two species.

Table 4. Pairwise Fst for population comparison in *M. angulata* (right diagonal) and *M. sikamea* (left diagonal)

	Pak Nai	**San Tau**	**Shui Hau**	**Tai Ho Wan**	**Yi O**
San Tau	0.011	-	0.105	0.059	0.052
Shui Hau	-0.019	-0.009	-	-0.036	-0.079
Tai Ho Wan	-	-	-	-	-0.043

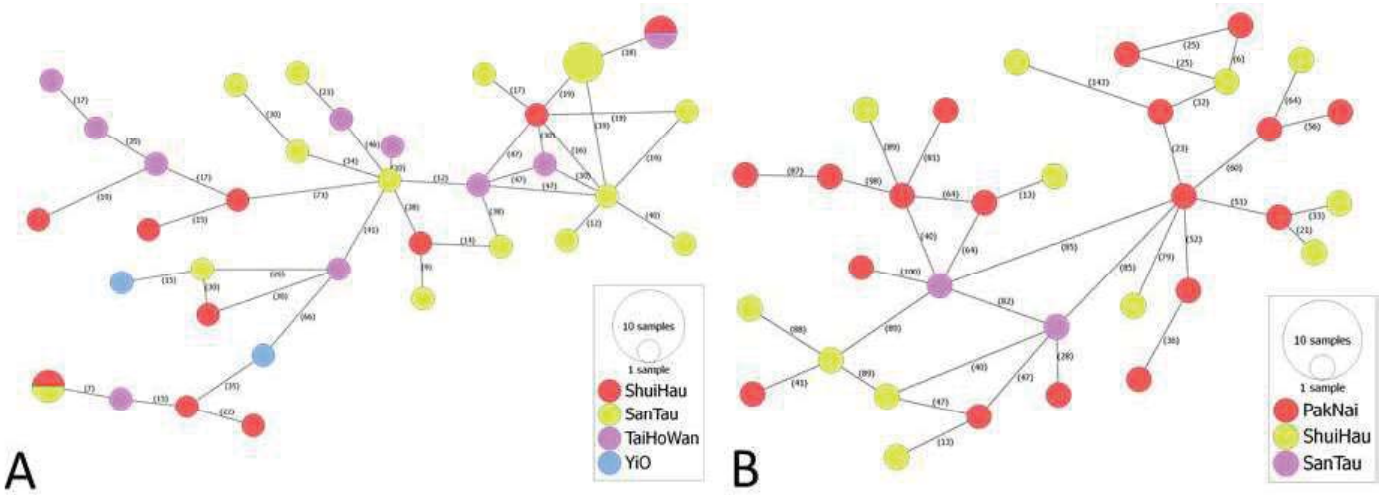

Figure 1. Minimum spanning haplotype network constructed from mitogenomic data of (A) *M. angulata* and (B) *M. sikamea*. Each coloured circle represents a haplotype and the area is proportional to the number of individuals bearing that haplotype. Numbers on the vertices indicate the number of mutation steps between haplotypes.

Discussion

Oysters are broadcast spawners with high fecundity and a long pelagic larval duration of around 20 days (Helm et al., 2004); coupled with a high early-life mortality rate, variance in the number of offspring contributed by each parent to the adult spawning population is expected to be high (Dennis Hedgecock & Pudovkin, 2011). Therefore, populations of broadcast spawning marine invertebrate species are often characterized by having high levels of genetic connectivity and sweepstakes reproductive success. Data collected for this study showed high haplotype diversity, low nucleotide diversity and lack of strong barrier to gene flow between populations of *M. angulata* and *M. sikamea* in western side of Hong Kong. This outcome agrees with the predictions based on other broadcasting sessile invertebrates, whereby there is high connectivity and little population structuring over the geographical scale of this study.

Since wild oyster populations in Hong Kong are genetically diverse, genetic rescue measures (Whiteley et al., 2015; this study) are likely to be unnecessary and future oyster reef restoration programs in Hong Kong should aim at using locally collected oyster spat to enable the best outcomes. In contrast, hatchery produced oyster spat often have a genetic diversity lower than the ones collected from the wild (Hornick & Plough, 2019) because of the targeted breeding for selected traits that characterizes breeding for aquaculture. In restoration programs in natural systems, this low genetic diversity might compromise the adaptive potential of the population, making the restored population less resistant and resilient to disturbances. Furthermore, oyster spat collected from other localities might have high genetic divergence from oyster populations in Hong Kong and could potentially be maladapted to local environmental conditions. As we have shown in this study, there is a large shift in relative abundance of different species across our sample sites, which may be related to environmental conditions, reproductive success, or post-settlement mortality. Therefore, care should be taken to avoid introducing foreign individuals to the local gene pool, hence minimizing the risk of introgression of maladaptive genetic variations. As the natural settlement of oysters in Hong Kong is generally high (Lau et al. 2020), restoration through support of the natural recruitment should be attempted in the first instance and hatchery-augmented restoration only attempted if natural settlement fails. Finally, as reported in Part 3 of this report, oyster reefs around Hong Kong have different oyster species compositions, and currently there is no efficient method to determine the species identity of the oysters; by using oyster spat collected locally from near to the restoration site, the risk of disrupting the natural oyster species composition should be minimized.

Part 5: Benthic biodiversity in an oyster reef

Introduction

Regular monitoring of ecosystems is an important practice in keeping track of the health trends of habitats; it provides the necessary information to policy makers for deciding if interventions are needed. To date, most ecosystem assessments were based on the morphological identification and quantification of a subset of species present in the habitat of interest (Baird & Hajibabaei, 2012). However, this approach has inherent flaws that limit its utility in the effective management of ecosystems. Even for skilled taxonomists, sorting through hundreds to thousands of specimens to species level and enumerating them is a time-consuming and resource intensive process which can take months to years to complete. In practice, to reduce the time and funding needed for sorting samples, identification is often done to coarse taxonomical levels, thereby limiting the resolution of the data, and potentially compromising on the robustness of the inferences generated. Environmental genetics has been proposed to replace traditional approaches in ecosystem monitoring (Cordier et al., 2020). With recent advancements in high-throughput sequencing technologies, it is becoming increasingly popular for conservation biologists to study ecosystems by examining DNA present in the environment. In the third part of this project, we demonstrated the use of environmental DNA amplicon sequencing as a viable tool for monitoring the biodiversity in oyster reefs, using the microbiome associated with reefs as the study system, and showed the potential for the technique to capture microhabitat biodiversity turnover.

Methods

Sediment samples were collected form the Tai Ho Wan mudflat (22°17'53.9"N 113°58'17.9"E). In order to capture the effect of oysters on sediment biodiversity, the sampling area was subdivided into four zones based on oyster density (Figure 3). The distance between zones was less than 10 meters, thus difference in environmental conditions should be negligible. To quantify the difference in oyster density between zones, ten 1 m x 1 m quadrants were haphazardly placed in each of the four zones. Oyster areal coverage in each quadrat was estimated by two independent observers; the averaged value of their estimated oyster percentage coverage was recorded. To quantify oyster density, a 0.25 m x 0.25 m quadrat was haphazardly placed inside each 1 m x 1 m quadrat. The number of oyster present inside the quadrats was then counted by two independent observers because of the high densities; the averaged value of the oyster count was recorded. Kruskal–Wallis tests were performed to test for difference in oyster coverage and oyster count between zones; Dunn's test was performed to test for pairwise difference between groups.

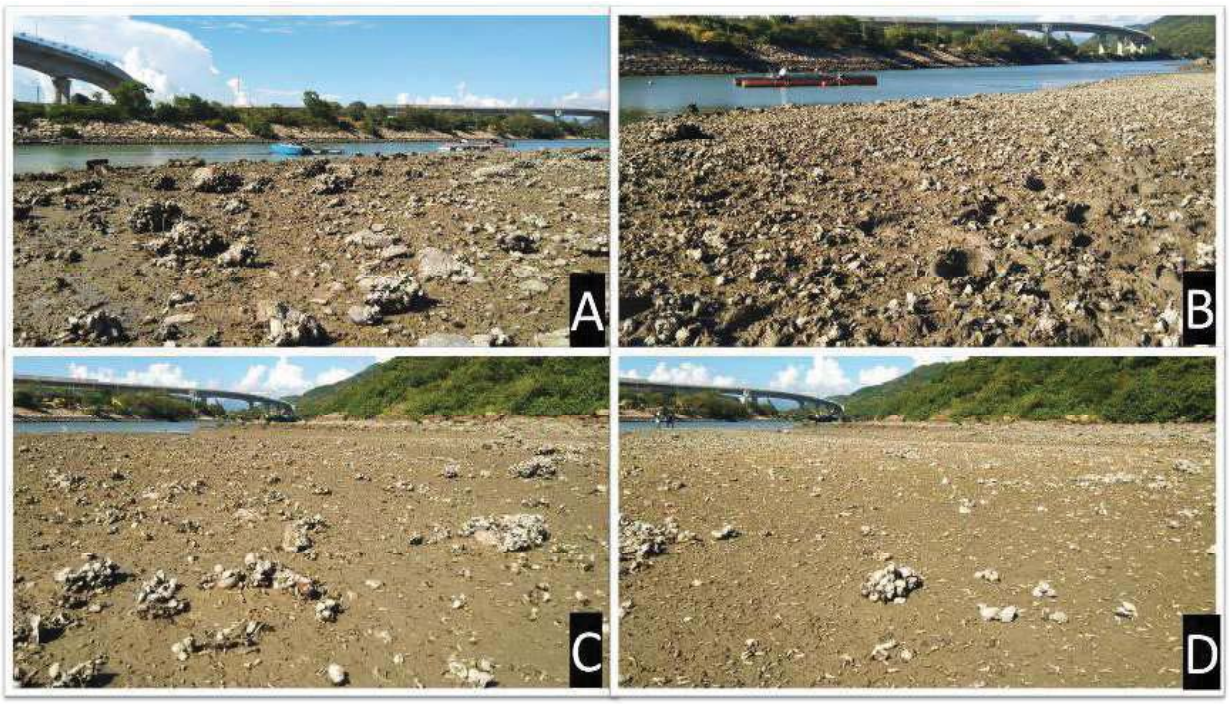

Figure 2. The sampling site. Zone A and Zone B had high oyster density while zone C and Zone D had low oyster density.

Six surface sediment samples were collected from each of the four zones. Sediment samples were stored in sterile polypropylene tubes and transferred back to laboratory on ice, then transferred to -80°C freezer for storage until DNA extraction within 4 hours from sample collection. Total DNA was extracted from 0.5 g of sediment sample using the DNeasy PowerSoil Pro Kit (Qiagen) following manufacturer's instructions, and subject to quality check with agarose gel electrophoresis (0.8%) and UV spectrometry (NanoDrop™ One, Thermo Scientific™), then stored in -20°C until further analysis. Two extraction blanks were included to quantify the level of contamination originated from the extraction kit or the extraction process.

Amplicon sequencing libraries targeting the 16S rRNA V4-V5 region and 18S rRNA V1-V2 region were prepared following the TaggiMatrix protocol (Table 5; Glenn, Pierson, et al., 2019). Two genetic loci were selected since each of them enables us to study different groups of organisms; the 16S rRNA gene is frequently used in the study of bacterial communities while the 18S rRNA gene is commonly used for studying eukaryotes. The targeted loci were amplified with the primer pairs 515F-Y/926R for 16S rRNA (Parada et al., 2016) and SSU_FO4/SSU_R22 for 18S rRNA (Blaxter et al., 1998); all primers included a variable length internal index (5-8 bp) an a fusion at their 5' end. The genes were amplified in 25 µL reactions with NEBNext® Ultra™ II Q5® Master Mix (New England Biolabs), following manufacturer's instructions. The reaction conditions were described in the table above. PCR products were purified with AMPure XP magnetic beads (Beckman Coulter) in 1:1 ratio and

inspected on 1.5% agarose gel. Two PCR blanks were included for each amplicon to monitor contamination originated from the PCR process.

Table 5. PCR conditions for amplicon amplification

	16S V4-V5	
Step	Temp	Time
Initial Denaturation	98°C	30 s
16 cycles	98°C	10 s
	62°C	15 s
	72°C	20 s
Final Extension	72°C	2 min

	18S V1-V2	
Step	Temp	Time
Initial Denaturation	98°C	30 s
22 cycles	98°C	10 s
	64°C	15 s
	72°C	20 s
Final Extension	72°C	2 min

Purified amplicons were pooled together in equal concentrations for a second round of PCR with iTru 8 nt indexed primers (Glenn, Nilsen, et al., 2019). The second PCR was performed in triplicate, in 50 µL reaction volume with NEBNext® Ultra™ II Q5® Master Mix (New England Biolabs) following manufacturer's instructions. The reaction conditions were as follows: initial denaturation of 30 s at 98°C; 8 cycles of 10 s at 98 °C and 75 s at 65°C; and a final extension step at 65°C for 5 min. PCR products were purified with AMPure XP magnetic beads (Beckman Coulter) in 1:1 ratio and inspected on 1.5% agarose gel. The amplicon libraries were then pooled with 6 other amplicon sequencing libraries and sequenced on Illimina MiSeq with MiSeq Reagent Kit version 3 (2 x 300 bp) in the Centre for PanorOmic Sciences, HKU.

Raw sequencing reads were adapter trimmed and demultiplexed with *cutadapt* v3.1 (Martin, 2011), and subsequently imported into *Qiime2 2020.11* (Bolyen et al., 2019). Read pairs were denoised and merged using DADA2 (Callahan et al., 2016), with the forward and reverse reads truncated to 290 bp and 210 bp respectively. In order to perform taxonomical classification of the sequencing reads, a feature classifier was trained on the Silva 138 99% OTUs full length sequences dataset; to improve the accuracy of the Naïve Bayes classifier, the dataset was subset so that only the region of the target sequences was used for training the classifier (Bokulich et al., 2018; Quast et al., 2013). Merged 16S and 18S read pairs were classified to genus or family level respectively with the respective classifiers, followed by a contingency based filtering to remove taxonomic groups which were only observed in one sample; this was done to minimize the risk of incorporating erogenous data derived from PCR or sequencing errors. Functional profile of the bacterial communities were predicted from the 16S rRNA sequence profiles with PICRUSt2 v2.3.0_b (Douglas et al., 2020). Diversity metrics for both taxonomical profiles were rarified to 78,900 reads and 95,500 reads per sample for 16S and 18S sequencing reads, and biodiversity metrics were calculated with the *diversity* plugin in Qiime2. ANCOM was used to detect for features that were differentially abundant across groups (Mandal et al., 2015).

Results

The summary for oyster coverage and oyster counts from the four zones were shown in figure 4; both oyster coverage ($H = 25.981$, $p < 0.001$) and oyster count ($H = 19.735$, $p < 0.001$) were different between the sampling zones. Dunn's test was performed to pinpoint which zones contributed to the difference in oyster count and coverage, and showed that both oyster count and coverage were higher in zones A and B than in zones C and D (Figure 4, Table 6).

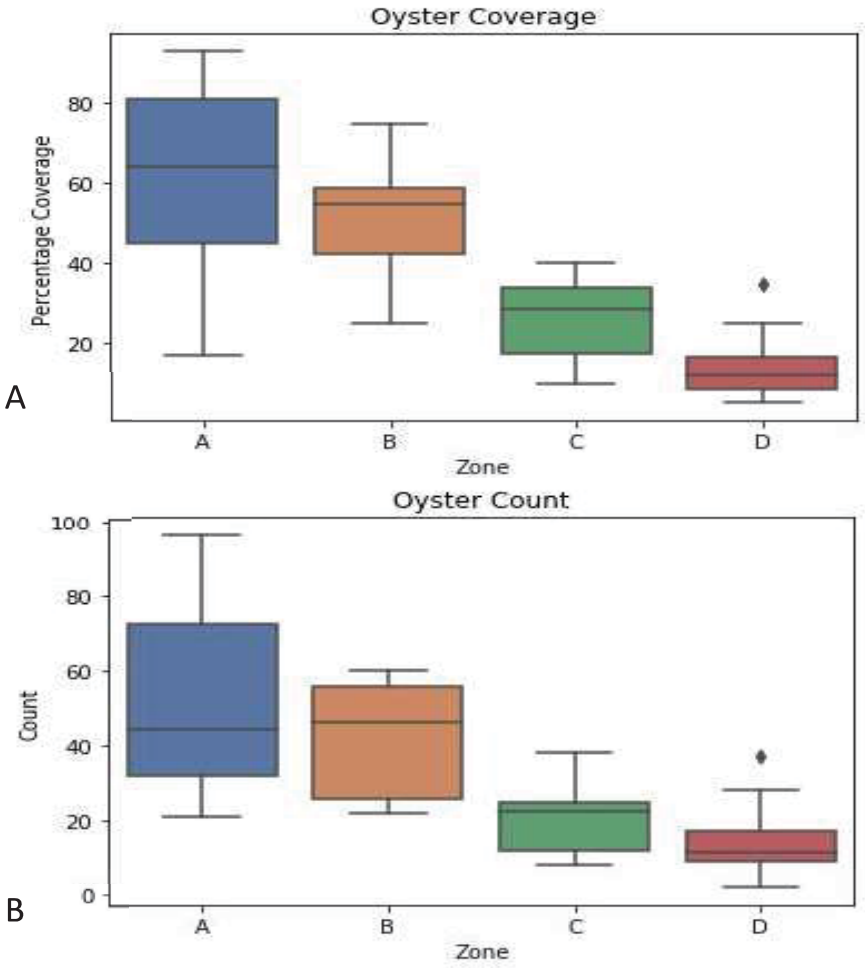

Figure 3. Oyster coverage (A) and density (B) in the four sampling zones.

Table 6. p-values from pairwise comparisons using Dunn's test. Upper diagonal corresponds to oyster coverage while lower diagonal corresponds to oyster count

	Zone A	Zone B	Zone C	Zone D
Zone A	-	0.618	0.002	< 0.001
Zone B	0.781	-	0.01	< 0.001
Zone C	0.006	0.013	-	0.192
Zone D	<0.001	<0.001	0.374	-

After demultiplexing, 16S amplicon data from three samples (one from zone B, two from zone C) were removed from downstream analysis due to low number of reads. After quality trimming, denoising and merging, an average of 113566 ± 28460 and 133779 ± 16687 non-chimeric reads were retrieved per sample from 16S amplicon and 18S amplicon sequencing data respectively. Average number of non-chimeric reads from blanks were 1437 ± 256 and 3608 ± 375 for 16S and 18S sequencing data; reads originated from contaminations were nearly two orders of magnitude lower than reads originated from samples, thus should not exert a strong effect on downstream analysis.

The non-chimeric 16S and 18S reads to genus and family levels respectively and alpha rarefaction plots were constructed to examine the adequacy of sequencing depth (Figure 5). In all samples, the rarefaction curve had commenced to plateau, indicating that the sequencing depth should be adequate to provide realistic estimate of true biodiversity.

Shannon index is a measurement of biodiversity that considers species richness, evenness, and dominance, and is one of the most popular biodiversity indexes. Kruskal-Wallis test was performed to test for difference in the Shannon diversity calculated from the samples and showed that zones with high oyster density housed higher bacteria diversity than zones with low oyster density ($p = 0.003$); eukaryotic diversity was similar between the zones ($p = 0.6744$) (Figure 6).

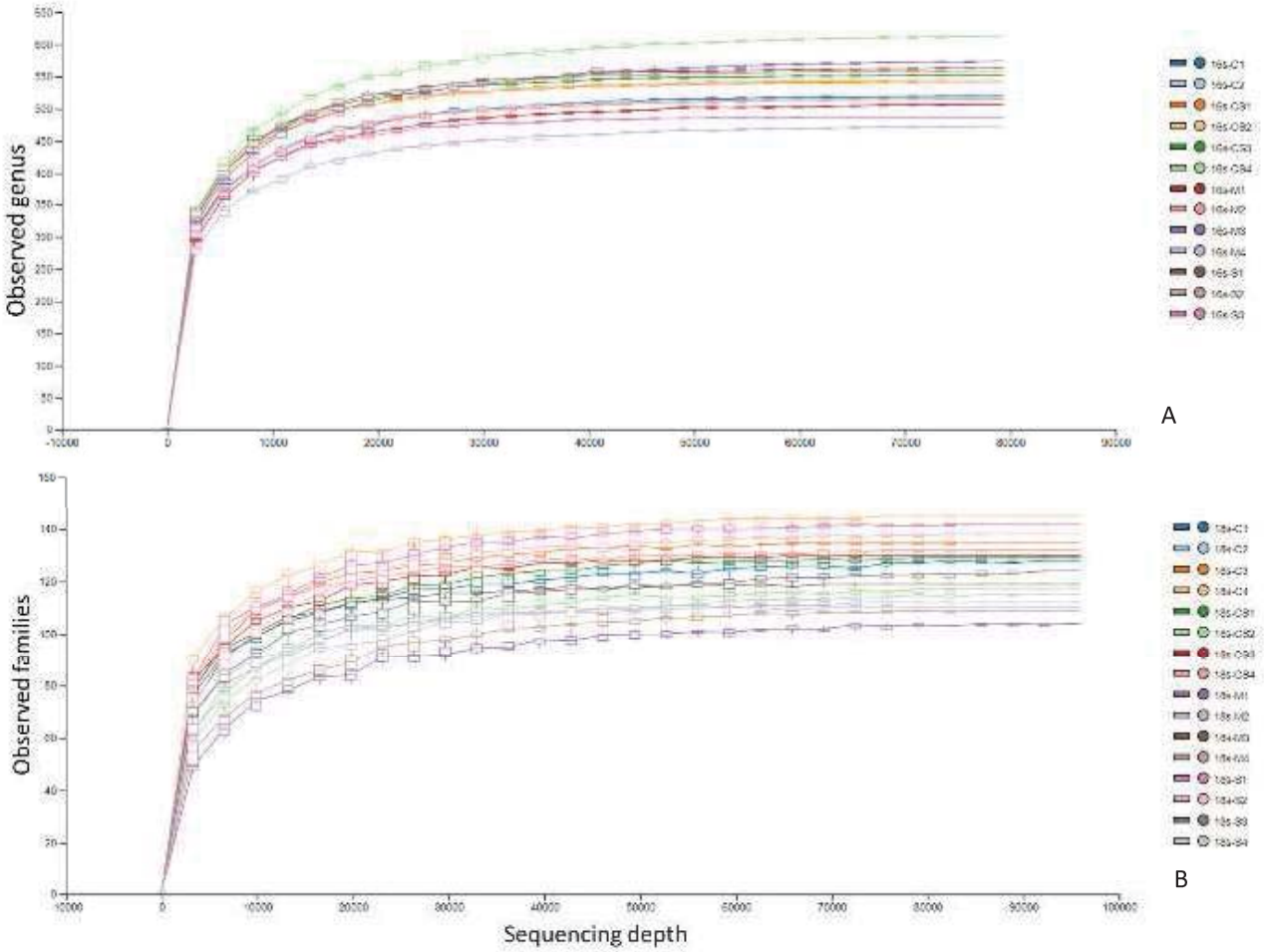

Figure 5. Alpha rarefaction plot for 16S amplicon (A) and 18S amplicon (B)

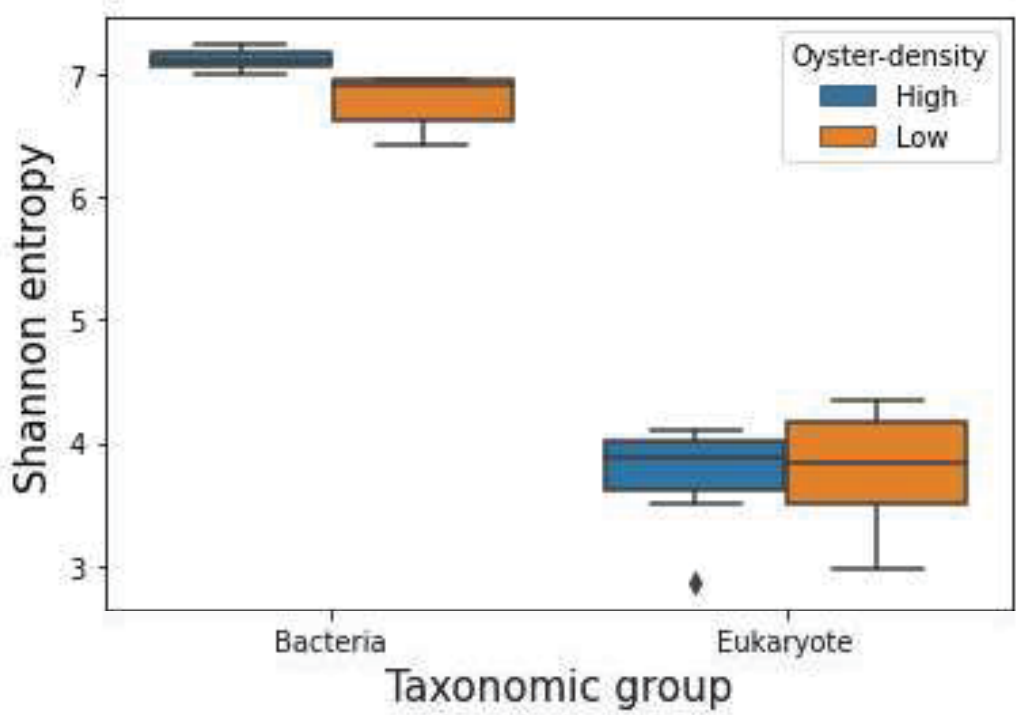

Figure 6. Shannon entropy for the two taxonomic groups in zones with different oyster density

To quantify the compositional dissimilarity in bacterial and eukaryotic community between zones with different oyster densities, the Bray-Curtis dissimilarity index was calculated. Difference in bacterial ($p = 0.003$, PERMANOVA) and eukaryotic ($p = 0.026$) community composition were detected between zones with different oyster densities (Figure 7). ANCOM results suggested that zones with high oyster density had a higher abundance of Ostreoida ($W = 66$) and *Hanstruepera* ($W = 562$), while zones with low oyster density had a higher abundance of Ploimida ($W = 83$).

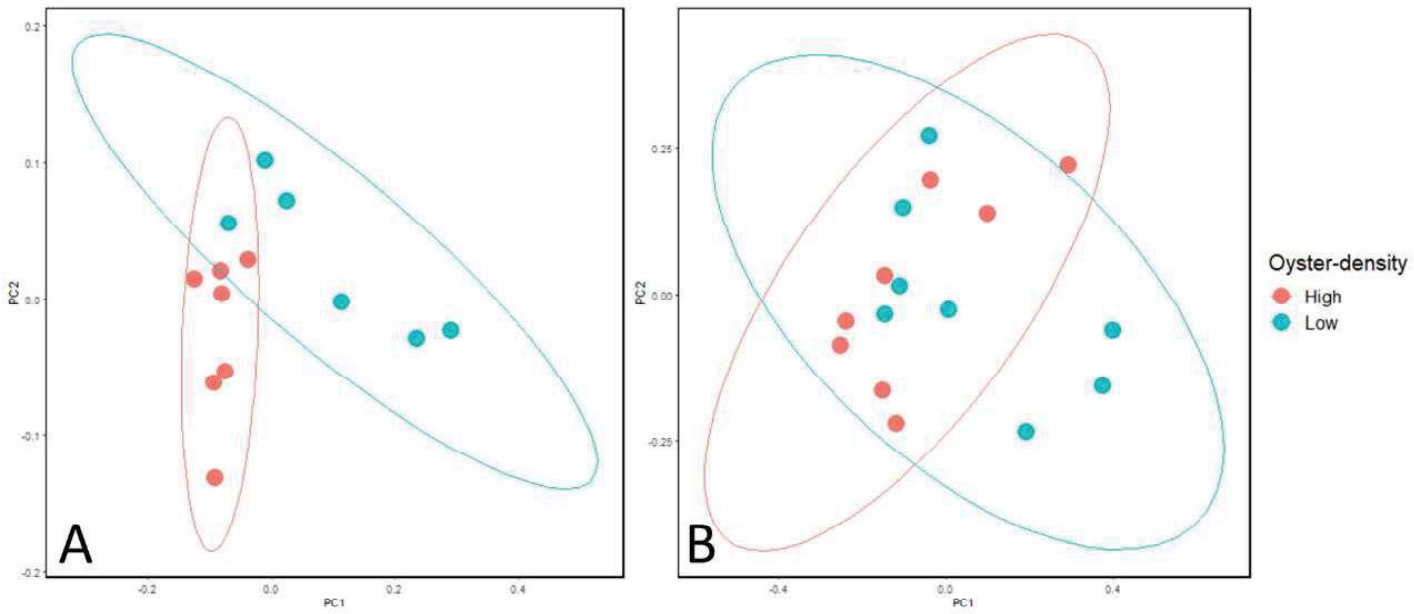

Figure 7. Principle coordinates analysis with 95% confidence ellipse of the bacterial (A) and eukaryotic (B) community composition in the sampling zones

PICRUSt2 was used to predict the functional abundance of abacterial community based on marker gene sequences. Shannon diversity and Bray–Curtis dissimilarity was calculated for the predicted functional abundance, and the bacterial community from zones with different oyster abundances were found to have similar functional diversity ($p = 0.12$, Kruskal-Wallis test) but different functional profile ($p = 0.003$, PERMANOVA). ANCOM was performed to identify the biochemical pathways that were contributing to the difference in functional profile and identified that denitrification pathways were roughly 1.6x more abundant in the microbial communities from zones with high oyster abundance ($W = 203$).

Discussion

Environmental DNA sequencing is becoming an increasingly utilized tool in biodiversity monitoring efforts. As a relatively new tool which is rapidly developing, these techniques have been mostly used to identify the species at one location of interest and to date are rarely used in the study of biodiversity turnover between microhabitats; this study provides a proof-of-concept that such techniques are perfectly viable tools for this purpose.

We found that habitat zones with high oyster density house a much more diverse bacterial community than zones with less oysters, and the composition between bacterial communities from the zones were different. This finding is not entirely surprising, as there is ample evidence supporting the proposition that oysters support benthic microbial activities, denitrification in particular, through biodeposition of organic rich materials (Higgins et al., 2013; Newell et al., 2002; Smyth et al., 2013). While perhaps not surprising based on published denitrification rates, quantifying this difference in microbial diversity, and the

different community, is the next step to understanding the processes which lead to enhancement of denitrification by oyster reefs. Interestingly, however, we only detected one bacterial genus (*Hanstruepera*) that had significant difference in abundance across habitat zones with different oyster densities, and the bacteria was likely to be a symbiont of oysters (He et al., 2018).

Despite having a higher taxonomical diversity in zones with greater oyster density, we did not detect an increase in bacterial functional diversity in zones with high oyster density. This finding adds support to the theory that high bacterial diversity within habitats or environments contributes to functional redundancy rather than functional diversity (Louca et al., 2018). Therefore, the greater diversity found within oyster habitats may lead to greater resilience in the functions performed by the microbial communities and maintain the associated ecosystem services under greater environmental stress (e.g., reduced water quality or climate change). Indeed, functional redundancy in microbial communities has been suggested to increase functional resilience against disturbances (Allison & Martiny, 2008), thus the potential for oyster reef restoration programs as a means to protect the proper ecosystem functioning of mudflat habitats under the face of climate change should not be overlooked. Nonetheless, in this study, functional diversity was inferred from taxonomical data and was not directly measured; follow-up studies are needed to confirm the robustness of this observation.

In contrast to bacterial communities, there was no pronounced difference in the diversity of eukaryotes between habitat zones with different oyster density, and the difference in community composition was moderate. This could be a result of the distance between sampling zones being small, and the range of motion of eukaryotes being generally much greater than bacteria. While we intentionally selected zones of different oyster densities in relatively close proximity to minimize any potential confounding by differences in environmental conditions, eukaryotes can readily move across larger distances than separating our habitat zones. However, if the distance between sampling sites were to be increased, the difference in eukaryotic diversity between zones might be more noticeable.

Microhabitat structure has historically been seldom considered in oyster reef conservation programs (Baggett et al., 2014). However, we found that microhabitat structure in oyster reefs exerts an influence on the biodiversity and microbial functional redundancy in the sediments which form the matrix of the habitat. Further studies are needed to examine the influence of oysters on sediment biodiversity across different spatial scales, and to identify whether the mode of influence is the same across seasons. Such information would be extremely valuable in advancing our mechanistic knowledge on ecosystem functioning in oyster reefs, thus enabling us to restore oyster reefs in a way that will enhance and support proper functioning of the habitat.

Part 6: Summary and recommendations

In this project, we examined the biodiversity of oyster reefs from three different perspectives, namely species diversity, genetic diversity, and biodiversity of the inhabiting community.

In the first part of the project, we found that contrary to popular belief, *M. hongkongensis* is not the dominant *Magallana* species in Western Hong Kong but rather *M. angulata* and *M. sikamea* are two of the most abundant. Furthermore, we detected a difference in oyster species composition among different sampling sites. Moreover, we exemplified the difficulty in accurately identifying the species identity of oysters by visual examination of shell morphology. The main implications of this part of the project are that: 1) care must be taken in oyster reef restoration programs to ensure that the proper species are used; and 2) DNA barcoding is advisable when accurate species identification is required. As an example of where this may become important, if spat of for oyster reef restoration are sourced from elsewhere in the Guandong province they may not be an appropriate species for the conditions at the restoration site. Therefore, restoration should be attempted with the local species in the first instance.

In the second part of the project, we found that the populations of *M. angulata* and *M. sikamea* in western Hong Kong were genetically diverse, and there is no strong barrier to gene flow among populations. Future oyster reef conservation efforts should aim at maintaining the high genetic diversity by: 1) avoiding the use of hatchery produced oyster spat which generally have low genetic diversity due to selective breeding; and 2) avoid the use of spat collected from sites that are distant from Hong Kong, as the spat might contain alleles that are maladapted to the environments in Hong Kong.

In the third part of the project, we found that the presence of oysters promotes sediment bacterial diversity, but the area of effect is quite small. This demonstrates the importance of microhabitat structure in the proper functioning of oyster reefs. Further research is needed to study the effect of oysters on sediment biodiversity over different spatial scales, which would in turn enable conservation practitioners to make better decisions when planning oyster reef restoration programs.

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