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Monitoring Bacterial Biodiversity in Surface Sediment Using Terminal Restriction Fragment Length Polymorphism Analysis (T-RFLP): Application to Coastal Environment

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Abstract—By using traditional bacterial culture methods, only less than 1% of bacteria from environmental samples can be cultivated for biodiversity and microbial ecology studies. In recent years, however, rapid development in molecular biology and ecology has enabled us to explore genetic diversity of bacteria in environmental samples using culture-independent approaches. For example, the recent emergence of (ribosomal DNA) terminal restriction fragment length polymorphism (T-RFLP) analysis has made it become feasible to investigate bacterial diversity more accurately, reliably, speedily and at cheaper cost in coastal sediment samples. This brief paper provides an overview of pros and cons of the molecular approach, T-RFLP, in analyzing the diversity of bacteria in surface sediments of contrasting coastal environments in sub-tropical region. We used the terminal restriction fragment length polymorphism (T-RFLP) analysis to track the changes of bacterial community compositions in surface sediments collected from five sites along a pollution gradient at six sampling occasions (2004 to 2006) in subtropical Hong Kong waters. Our application of this technique in coastal environments illustrates the feasibility and the power of this novel approach for environmental monitoring and ecosystem recovery studies.

Keywords: surface sediment, Victoria Harbour, sewage pollution, bacterial community, T-RFLP

1. INTRODUCTION

Surface sediment bacteria play a significant ecological and biogeochemical role in marine ecosystems due to their high abundance relative to the overlying water column and they play a key role in the decomposition of the organic matter, nutrient cycling and carbon flux. Despite their importance, our knowledge of the bacteria that inhabit surface sediments is very limited, especially in the heterogeneous marine ecosystems such as Hong Kong (Wu et al., 2004). It is long since known that dense aggregations of bacteria may colonize clean surfaces of both hard and soft substrates in the sea quite

rapidly, following adsorbed organic molecules which may serve as a stimulus for the initial immigrants: bacteria, fungi, and unicellular algae. Bacterial communities are structured by temporal and spatial variability of physicochemical and biotic parameters (Hewson et al., 2007). Importantly, bacterial communities readily respond at extremely faster rates (compared to other benthic organisms) to environmental and pollution changes. Thus reflect their micro environmental conditions and "communicate" this information to other biota in their vicinity and play key role in benthic-pelagic coupling. The estimation of bacterial abundances as well as their genetic diversity under *in situ* conditions is therefore the most fundamental objective of aquatic microbial ecology.

The detection of bacterial diversity and their sptaio-temporal variation in surface sediments is also of great practical and scientific relevance, especially in coastal ecosystems. Recently, the analysis of changes in surface sediment bacterial community has been used for detecting and monitoring the biological effects of human activities in the marine environment (Zhang et al., 2008b). If the bacterial community structure in soft-benthic habitat is determined by their environment, then pollution loading or organic enrichment is expected to shift their composition, and a counter shift toward the original community should be evident after the abatement of pollution discharge (Yoza et al., 2007). But the response of bacterial community in sediments to anthropogenic disturbances and environmental gradients has been rarely investigated; primarily due to technical challenges associated with cultivation of sediment bacteria (see Bissett et al., 2006). In the past, quantitative and qualitative analyses of microbial communities have been hindered by the inability to cultivate most of the bacteria originally isolated from natural samples. Recent developments in molecular finger printing techniques, however, have provided new insights into our understanding of variation in bacterial diversity in natural samples, and their response to environmental heterogeneity. Thus these modern tools have provided powerful alternative to the culture-dependent techniques. For instance, bacterial community analysis by terminal restriction fragment length polymorphism (T-RFLP) analysis has been shown to be effective at discriminating between bacterial communities in environmental samples including coastal sediments (Zhang et al., 2008b; Tsoi et al., under review).

Today, a major scientific challenge is to evaluate the significance of the great diversity of bacterial life in surface sediments. Surface sediment bacteria play a significant ecological and biogeochemical role in soft-bottom marine ecosystems, including tidal flats, due to their high abundance relative to the overlying water column. Despite their importance, our knowledge of the bacteria that inhabit surface sediments is very limited, especially in the heterogeneous marine ecosystems. Therefore, this paper summarizes our current knowledge on bacterial community diversity, abundance and distribution on surface sediment in contrasting coastal environments. Current progress in this field is exemplified using recent and ongoing research from our group. Specifically we provide, 1) an overview of molecular fingerprinting method, the T-RFLP, used to study bacterial community diversity, 2) a comparison of bacterial community structure in contrasting coastal environments at various spatial and temporal scales, and 3) a discussion of how the molecular

fingerprinting techniques will improve our understanding of bacterial diversity and their application in environmental monitoring and ecosystem recovery studies.

2. SURFACE SEDIMENT BACTERIAL COMMUNITY

Surface sediment bacterial community is a major component of microbial food webs, biogeochemical cycles and energy flow. Their biodiversity is structured and determined by the temporal and spatial variability of physicochemical and biotic parameters and thus, can reflect local environmental conditions (Urakawa et al., 1999; Zhang et al., 2008a, b). Therefore, any shift in nutrient, environmental and pollution profiles in the benthic-pelagic ecosystems will directly impact bacterial community that in turn further affects nutrient cycles and other related communities. Besides, bacterial communities are vulnerable to natural and anthropogenic disturbances such as global climate change, pollution, heavy metal contamination, organic pollution and enrichment. For example, coastal pollution (DDT and heavy metals) substantially reduced bacterial diversity and suppressed their activities in surface sediments (Han et al., 1999; Gillan et al., 2005; Duran et al., 2008). Different types of bacteria living in sediments, including aerobic heterotrophs, chemolithotrophs, more specifically, hydrogen oxidizing bacteria, sulfur-oxidizing bacteria, iron-oxidizing bacteria, nitrifying bacteria, methanotrophs, fermentative bacteria, nitrate-respiring bacteria, metal-respiring bacteria, sulfur- and sulfate-reducing bacteria, methanogens, acetogens, and syntrophic bacteria (Zhang et al., 2008b).

Bacteria in surface sediments attract other biota by providing resources (e.g. food, habitat, shelter), or, by signaling settlement sites with increased fitness expectations (i.e. high survival probabilities until reproduction) and, conversely to avoid others with low fitness potential. Now, methodological break-through allow to genetically identify and measure bacterial diversity and abundances, and demonstrate their functional capabilities, so in the production of allelochemically deterrent, toxic, or biocommunicative compounds mediating the settlement of invertebrate larvae. Consequently, the interactions of bacterial communities and settlement chemical cues became a focal point of research in the context of supply side ecology and marine invertebrate larval settlement (see Thiyagarajan et al., 2006). Larvae of benthic marine invertebrates recognize suitable settlement substrata by using various environmental cues, including biofilms composed of complex matrices of macromolecular deposits and microbial constituents. Thus, bacteria are key players in the marine sediment ecosystem as they directly affect many aspects of sedimentary microenvironment.

3. T-RFLP

Over the last decade, the understanding of bacterial diversity and dynamics in marine sediments has significantly increased due to the rapid development of culture-independent molecular methods that allowed us to obtain more detailed information on the phylogeny and distribution of non-cultivable microorganisms (Amann et al., 1990). Particularly, the T-RFLP has been considered as a powerful tool for assessing species richness and the population sizes of various species in the complex bacterial

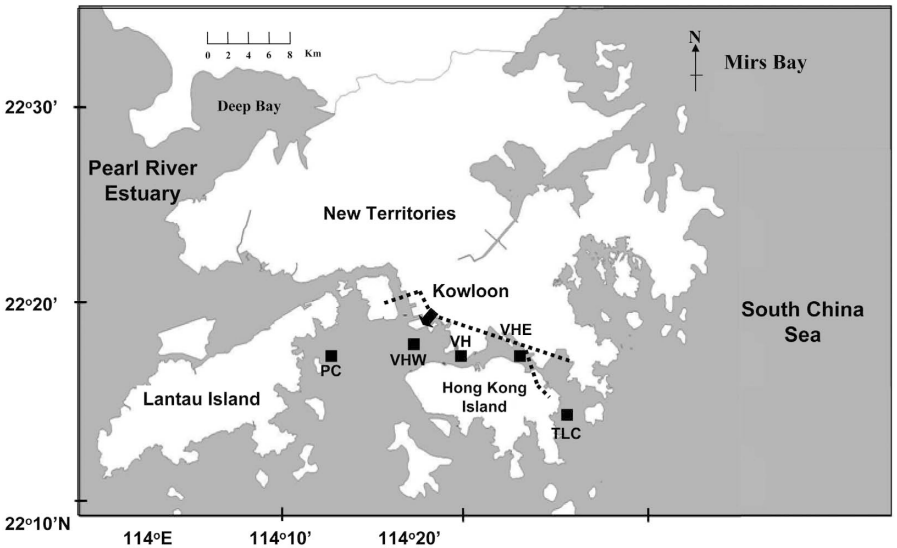

Fig. 1. The working method, principle and workflow of the terminal-restriction fragment length polymorphism analysis (T-RFLP).

community (Liu et al., 1997). With the T-RFLP method, after community DNA is isolated from a field sample, DNA coding for 16S rRNA is specifically amplified by PCR using primer pairs designed from the conserved region of the gene. The PCR product is then digested by a restriction enzyme and the length profile of terminal restriction fragment (TRF) labeled by the fluorescent dye is detected for the identification of species composition of the bacterial communities. The ratios of each PCR amplicon, estimated by measuring fluorescence emission intensity, could indicate the relative abundance of bacterial species. This method can be used to estimate the relative abundance of dominant bacterial species (relatively quantitative) and to study the pattern shift (in terms of dominant bacterial groups and their relative abundance) of bacterial community structures. The simple workflow of T-RFLP analysis is shown in Fig. 1.

Pros of T-RFLP analysis

It is well known that the cultivation of bacteria generally suffers from the fact that <1% of bacterial species in the marine environment are culturable and that bacteria are largely indistinguishable by morphological characteristics (Eilers et al., 2000). Therefore, investigations based on conventional techniques are incomplete and often bear a strong speculative element. Among a variety of molecular tools, we stared with T-RFLP. This method is rapid and highly automated, but does not allow the identification of bacteria in the samples (Lau et al., 2005).

Limitations of T-RFLP analysis

Data of T-RFLP analysis on bacterial community profile can be interpreted as "semi-quantitatively" according to the number of peaks in each sample (i.e. number of distinguishable bacterial types) as well as qualitatively according to the position of peaks (i.e. occurrence of unique bacterial types). T-RFLP data can only be regarded as "semi-quantitative" since one peak may represent many species of bacteria that share the same cutting sites for the restriction enzyme of experimenters' choice (Zhang et al., 2008a). The same as all other molecular tools, minority bacterial populations may not be detected by T-RFLP analysis since template DNAs from these populations represent a small fraction of the total extracted DNA and may not be amplified by PCR due to kinetic bias (Liu et al., 1997).

DNA fingerprinting techniques are valuable tools for the characterization of environmental bacterial communities, but different techniques have different limitations and biases. T-RFLP cannot differentiate closely related DNA sequences, which are likely to have the same terminal restriction site, and thus may reduce the number of detectable OTUs. However, T-RFLP is also prone to overestimation of bacterial diversity due to pseudo-TRF formation (i.e. single stranded amplicons that are recalcitrant to restriction enzyme digestion) (Egert and Friedrich, 2003). In contrast, DGGE can resolve closely related DNA sequences better than T-RFLP (Casamayor et al., 2002), but is more affected by heteroduplexe formation and rRNA operon heterogeneity (Muyzer et al., 1993), which can increase the number of OTUs artificially. Other possible biases are due to differential extraction of DNA from different bacterial populations (Martin-Laurent et al., 2001) as well as preferential PCR amplification of numerically dominant DNA templates (Polz and Cavanaugh, 1998).

4. MATERIAL AND METHODS FOR T-RFLP ANALYSIS

Description of study site

Victoria Harbor is one of the world's deepest natural harbors with 0.5–4.5 km in width and 4–40 m deep. A distinct hydrological feature of the Harbor is that it is mainly tide-controlled and has a relative large flushing capacity. It is estimated that 40% of water in the Harbor is exchanged daily, but 50% of the pollutant-associated particles discharged into the Harbor are deposited in its sediment. It is a contrasting environment and diverse in ecological conditions because it is generally polluted and located between the Pearl River Estuary in west side and the South China Sea in east (Fig. 2). It offers contrasting environments for comparative studies of microbial communities in response to environmental changes as this harbor is under heavy influence of the Pearl River discharge and oceanic flushing as well as domestic and industrial sewage discharge (HKEPD, 2005).

The environmental condition in the harbor experiences a rapid transition from an estuarine habitat in its west end to a heavily polluted area in the mid part and to the oceanic environment in its east end, all within 30 km. Because of the uncontrolled disposal of domestic and industrial wastewater since 1900, especially from the 1960s

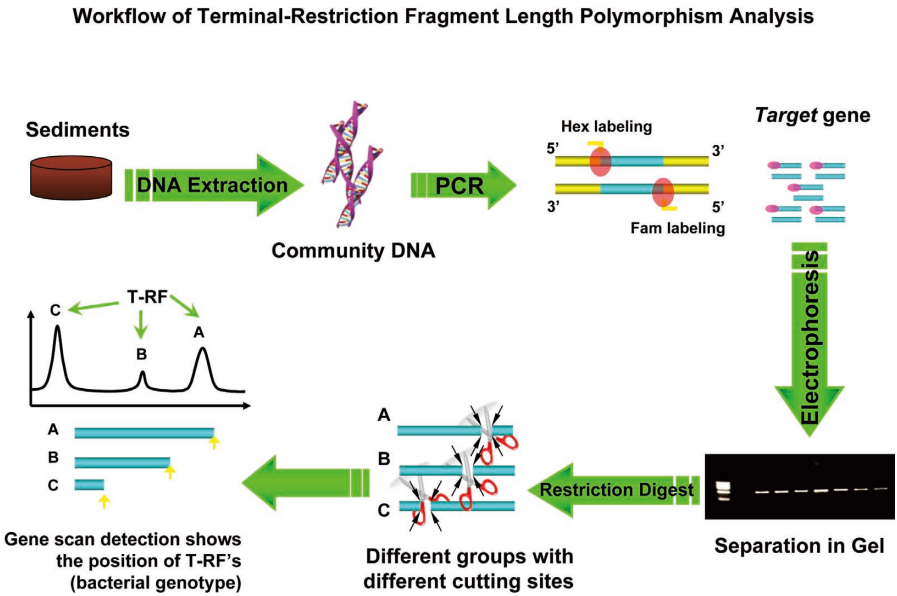

Fig. 2. A map showing the locations of sediment collection sites in Victoria Harbour, Hong Kong. TLC, Tung Lung Chau; VHE, Victoria Harbour east; VH, Victoria Harbour; VHW, Victoria Harbour west; PC, Peng Chau. The arrow shows the sewage discharge point.

to the 1980s, the harbor has become seriously contaminated (Wong et al., 2000). In the past, the wastewater from surrounding urban areas was discharged directly into the harbor after simple screening. For example, the harbor received and estimated 1.5 Mt of sewage and industrial wastewater per day in 1995 (Hong Kong Government, 1995). As a result, the harbor is heavily contaminated with bacteria (Yung et al., 1999), heavy metals (Tanner et al., 2000; Shi et al., 2007), and organic pollutants (Connell et al., 1998).

To protect Victoria Harbor environment, a large sewage pollution reduction program Harbor Area Treatment Scheme (HATS) has been undertaken in 2001. Approximately 75% of sewage loading into the harbor have been collected and discharged via pipelines to Stonecutter Island after further treatments. It is expected that the ecosystem in Victoria Harbor will gradually recover after the abatement of sewage discharge. The values of various geochemical parameters in sediments already showed a slightly decrease within a three year period, for example acid volatile sulfide (AVS), Electrochemical Potential, sulfate, and some of the heavy metals (Shi et al., 2007; personal communication with Dr. Xiang-Dong Li).

Sediment sample collection

Sediment samples collected from five sites along Victoria Harbour, Hong Kong, were used in this study to describe the application of T-RFLP method. These sites

were hereafter referred to as TLC (Tung Lung Chau), VHE (Victoria Harbour east), VH (Victoria Harbour), VHW (Victoria Harbour west) and PC (Peng Chau) (Fig. 2). All these sites are located at relatively stable depths (7 to 15 m). The western site (PC) is heavily affected by the discharge from the Pearl River and the eastern site (TLC) is oceanic. The transition sites, VH, VHE and VHW, are polluted by domestic sewage (treated and/or untreated) discharges from about 3.5 million people. Samples were collected during six sampling dates: summer 2004 (13-08-2004), winter 2004 (14-12-2004), summer 2005 (17-07-2005), winter 2005 (12-12-2005), winter 2006 (10-12-2006) and summer 2006 (21-06-2006). To obtain surface sediments of similar grain size, extra care was taken in selecting the precise sampling locations within each site. In each sampling, four homogenous surface sediment samples (from top 1 cm) per site and sampling date were collected using Gravity Core (KC DenMerk). Samples were transport back to the laboratory in dry ice and kept at –80°C until the extraction of total genomic DNA.

DNA extraction

Total DNA was extracted from the sediment samples according to Fortin et al. (2004) with some modifications. Approximately 0.4 g sediment samples was transferred into 2 mL screw cap microcentrifuge tube together with 0.1 g of glass beads and washed sequentially in three washing solutions followed by vortex mixing and centrifugation. The supernatant was discarded and the washed sediment sample was homogenized in 0.4 mL of extraction buffer on mini-beater. The cells were lysed by using three freeze-thaw cycles and chemical approach. The total DNA was extracted by twice with an equal volume of chloroform-isooamlyalcohol (24:1 vol/vol). The aqueous phase was collected and the DNA was precipitated and washed using isopropanol and 70% ethanol, subsequently. The extracted DNA was dissolved in dd H_2O and frozen at –20°C until further analysis.

PCR amplification of the 16S rRNA gene

PCR of the bacterial 16S rRNA gene (rDNA) in the crude DNA extract was performed in a total volume of 25 μL containing 0.1–0.2 μg of DNA template, 200 μM of each of deoxynucleoside triphosphate, 0.75 U of DNA Taq polymerase (Takara Biotechnology, Japan) and 0.2 μM of each of the domain specific primers: 8F (labeled at the 5′ end with a 6-carboxy fluorescen (FAM) dye) and 1055R. PCR was performed in the following thermal cycles: 95°C for 2 mins; 10 touchdown cycles of 95°C for 1 min, 60°C (reduced to 51°C in increments of 1°C $cycle^{-1}$) for 1 min and 72°C for 1 min; additional 15 cycles with a constant annealing temperature of 45°C; and 72°C for 5 mins. Successful amplification of DNA was verified by electrophoresis of 3.5 μL of PCR products in 1.5% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0).

T-RFLP analysis of bacterial communities

PCR products were cleaved with 10 U of the restriction enzyme Hae III at 37°C for 6 hrs. Cleaved PCR products were purified by ethanol precipitation and dissolved

in 20 μL of sterile dd H_2O. Ten μL of purified products together with 0.5 μL of internal standard (ET900R, Amersham) were denatured at 95°C for 2 mins, snap cooled on ice, and subject to electrophoresis on a MegaBACE genetic analyzer (Amersham) operated in the genotyping mode. After electrophoresis, the size of the fluorescently labeled terminal restriction fragments (TRFs) was determined by comparison with the size standard (ET900R, Amersham) using the software Genetic Profiler (Amersham). TRFs that were <35 bp and >900 bp in size were excluded from statistical analysis in order to avoid pseudo-TRFs derived from primers and inaccurate size determination respectively.

Statistical analysis of T-RFLP data

Bacterial community data (T-RFLP profiles) were analysed using a multivariate technique (see Qian et al., 2003 for details). Formal significance tests for differences among sampling dates and sites were performed using the crossed two-way ANOSIM (analysis of similarities) randomization/permutation test from the similarity matrix based on Dice coefficient, it was calculated based on the total number of TRFs observed in all samples and the presence or absence of these TRFs individual samples (Nakano et al., 2008). Multidimensional scaling (MDS) ordination was subsequently used to map the spatio-temporal relationships in the similarities for all five sites and six sampling dates. Pairwise comparisons of bacterial community composition between any pairs of sites in each sampling date were determined using ANOSIM randomization permutation test with 5000 permutations (Clarke and Warwick, 1994). The computer package PRIMER was employed for all non-metric ordinations in this study.

5. SPATIO-TEMPROAL VARIABILITY OF BACTERIAL DIVERSITY IN VICTORIA HARBOR

As described in the previous sections, the Hong Kong's coastal environment presents an excellent opportunity for marine microbiologists to explore the diversity and recovery of sedimentary microbes in relation to anthropogenic pollution and environmental gradient. The T-RFLP analysis was used to track the changes of bacterial community compositions (BCC) in coastal surface sediments along environmental, pollution and sediment biogeochemical gradients between 2004 and 2006 (see Fig 2 for sampling sites).

Generally, the multi-dimensional scaling (MDS) ordinations of T-RFLP profile of BCC for each sampling date revealed a site-specific pattern. For instance, BCC of VH formed a separate group on the lower right side of the MDS ordinations (Fig. 3). The BCC of the remaining four sites were not grouped tightly. Such MDS grouping of BCC were largely supported by the ANOSIM results (data not shown). Thus, our study clearly indicate that the BCC respond to environmental and pollution gradients of the study site.

Our study highlighted that BCC at the eastern site (TLC) shared a significantly higher similarity with those at the western site (PC) in all our sampling dates (see Fig. 3). These two relatively clean sites, however, these two sites had contrasting

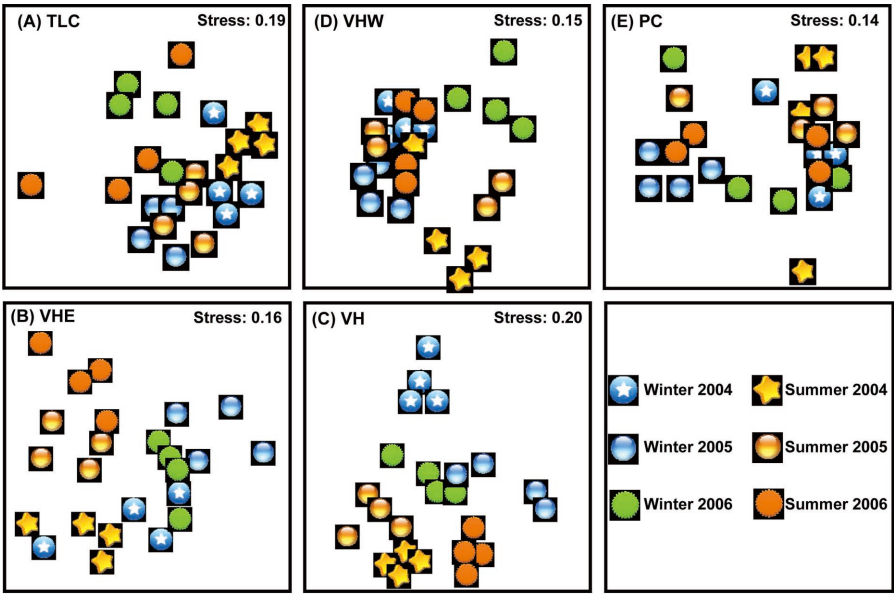

Fig. 3. Multi-dimensional scaling (MDS) ordinations showing the spatio-temporal variations of the bacterial community compositions in sediments collected from five sites (A–E). See Fig. 1 for site abbreviations.

environmental characteristics. Consequently, substantial seasonal shift in BCC was expected as different environments (salinity and productivity levels) presumably have different control effects on BCC. Surprisingly, however, there was not clear temporal pattern in BCC in these two control sites (Fig. 3). In contrast, the mid-harbour site, VH, had distinct BCC. Previous studies reported alterations in BCC in response to hydrocarbon (Cuny et al., 2007), zenobiotics (Hogan and Ward, 1998), and heavy metal (Ford et al., 2005; Gillan et al., 2005) contaminations. At the same time, BCC at the five sites showed different temporal patterns (Fig. 4). For example, there was a significant overlap between winter and summer samples collected from TLC, VHW, and PC. In contrast, the BCC in winter were distinctly separated from those in summer at VHE and VH.

6. PPLICATION OF T-RFLP IN ECOSYSTEM RECOVERY STUDY

Polluted vs. Control site

In this study, the VHW represented a sewage discharge site and PC served as a control site (Fig. 4A). In order to verify if there was been an impact of sewage discharge on bacterial community in surface sediments, the sediment bacterial communities compositions from these two sites were analysis by T-RFLP analysis and compared by using a pairwise comparison test, ANOSIM. Except in summer

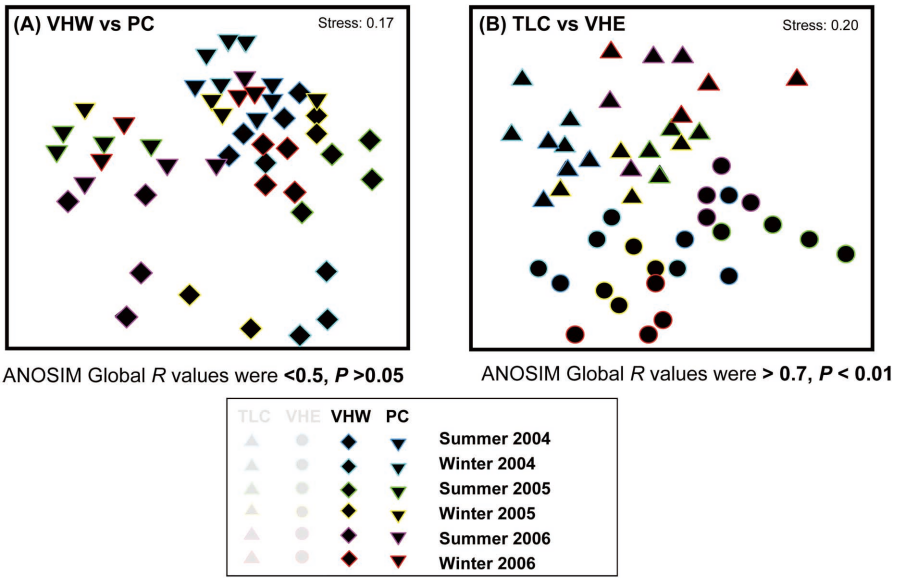

Fig. 4. MDS ordinations comparing the sediment bacterial communities between (A) sewage discharge site (VHW) and control site (PC) over six sampling dates, and (B) sewage abatement site (VHE) and control site (TLC) over six sampling dates. See Fig. 1 for site abbreviations.

2004 and winter 2005, the pairwise comparisons of the bacterial communities from VHW and PC showed low *R* values, indicating a high community resemblance to the reference site, PC (ANOSIM Global *R* values were <0.5, $P > 0.05$). The same trend was observed in the MDS ordination. Thus, there was no sign of adverse changes in the surface sediment bacterial community as a result of treated sewage discharge at VHW yet.

Pollution abatement and Control site

In this study, the VHE represented a sewage abatement site and TLC served as the control site (Fig. 4B). In all the six sampling dates, the pairwise comparisons of the sediment bacterial communities from TLC and VHE showed high *R* values, indicating the least community resemblance to the control site, TLC (ANOSIM Global *R* values were >0.7, $P < 0.01$). The same trend was observed in the MDS ordination, in which TLC samples grouped at the upper left side, irrespective of sampling date and the VHE samples at the lower right side. Complete recovery of bacterial community was not detected at VHE even after 5 years of sewage abatement. The lack of similarity in the bacterial community compositions between TLC and VHE was unexpected as the discharge of large amount of untreated sewage has been dramatically reduced since 2001. Noticeably, water quality (i.e. low number of *Escherichia coli*, nutrient content and Chl *a* levels) at VHE has improved significantly

after the implementation of the Harbour Area Treatment Scheme (HATS) in 2001 and the toxic metal discharges into the VHE have also been reduced. As a result, several sedimentary biochemical parameters and pollution levels have shown a decreasing trend (Li XD, personal communication). However, long-term simultaneous monitoring on sediment bacterial community, biochemical characteristics and pollution loading is required to address the bacterial community recovery at VHE.

7. SUMMARY AND FUTUTRE RESEARCH DIRECTIONS

The T-RFLP was used to track the changes of bacterial community compositions (BCC) in coastal surface sediments along a pollution gradient between 2004 and 2008. BCC in the chronically contaminated sites, VH, showed the largest deviation from that in the adjacent sites. Surprisingly, BCC at two contrasting environments (oceanic vs. river-influenced) shared higher similarity. Unexpectedly, the BCC did not recover even after 5 years of pollution abatement initiatives in one of the study sites. On the other hand, disposal of treated sewage for 5 years in one of the sites had an insignificant effect on BCC. A striking seasonal variation of BCC was observed only at the polluted sites, VH. Although factors other than pollution gradients may also explain the observed BCC patterns, the information presented here can be useful in predicting long-term effects of pollution on BCC. Overall, this is the first study providing the spatio-temporal analysis of surface sediment BCC in these contrasting marine environments and one of few in soft sediments. It seems, our three-years of BCC monitoring are not sufficient to trace the correlation between environmental gradient and BCC. In order to have a better understanding of the relationships among pollution, amendment processes, and bacterial communities, a longer monitoring time is needed.

In this study, T-RFLP technique has been shown to be sensitive and effective for detecting changes in bacterial community composition in response to different kinds of environmental and anthropogenic disturbances. Coupling molecular tools with traditional measurements of physico-chemical parameters and benthic macro fauna analysis thus, allows us to evaluate the response of benthos to anthropogenic disturbances. Among a variety of molecular tools, we started with T-RFLP due to its simple operation with an automatic genetic analyzer. Nevertheless, T-RFLP analysis does not allow the identity of bacteria in the samples. Investigators who are interested in the identification of bacteria in samples need to choose other techniques such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993) that allows the extraction and sequence of specific bands resolved on the gel.

Relatively, very high bacterial diversity was observed in our study using T-RFLP, the non-cultivated molecular methods. This information triggering us hypothesize that a large numbers of potentially a novel bacterial species harboring in the subtropical marine sedimentary environments. Therefore, substantial efforts in the future should target on identifying those new bacterial species via traditional culture methods. Furthermore, bacterial activity experiments on these potential novel species are also suggested. Many of these bacterial species may be potential pollution bio-indicators. Our future study should target on these specific functional bacterial

groups to reveal their roles in ecosystem recovery processes and to explore potential application of these bacteria on environmental pollution monitoring.

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