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Bacterial diversity in various coastal mariculture ponds in Southeast China and in diseased eels as revealed by culture and culture-independent molecular techniques

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Abstract

Mariculture ponds are widely distributed in Chinese coasts and have become a threat to the health of coastal ecosystems. In order to improve our understanding on the microbial composition in mariculture environments, we sampled a variety of ponds farming different animals or plants around the Dongshan Island and Xiamen Island in Southeast China and isolated cultures from the tissues of diseased eels. Analysis by polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE), clone library and direct culturing methods revealed highly diverse bacterial communities in these samples. Bacterial communities in the Dongshan samples were dominated by Alphaproteobacteria, Gammaproteobacteria and Bacteroidetes. The *Gracilaria verrucosa* pond harbours the most abundant species (20 DGGE bands), followed by *Epinephelus diacanthus* pond (18 bands), *Haliotis diversicolor* supertexta pond I (18 bands) and *Penaeus vannamei* pond (11 bands). In comparison with surface waters, *Penacus orientalis* pond sediment showed a much more complex bacterial community, from which only sequences affiliated with Deltaproteobacteria, Firmicutes, Acidobacteria and candidate phylum TM6 were found. Bacterial cultures in diseased eels were closely related to two pathogenic genera, *Aeromonas* in Gammaproteobacteria and *Bacillus*, in Firmicutes. Clones affiliated with another two genera, *Escherichia* and *Vibrio*, that have pathogenic potentials were also identified. Phylogenetic analysis of a total of 131 sequences showed that 48.9% of the sequences were clustered into Gammaproteobacteria and formed the most abundant group, followed by Alphaproteobacteria (19.1%), Firmicutes (7.6%), Bacteroidetes (5.3%), Deltaproteobacteria (5.3%), Actinobacteria (4.6%), Chloroplast (3.8%), Acidobacteria (2.3%), Cyanobacteria (1.5%), Betaproteobacteria (0.7%) and TM6 (0.7%). 43.7% (28/64) of the phylogenetic clusters cannot be classified into any known genus and 44.3% (58/131) of the sequences show $<95\%$ similarity to public database records, suggesting that abundant novel species exist in mariculture ponds. Gathering bacterial diversity data in mariculture ponds and diseased fish is meaningful for the prevention and control of fish diseases and for the improvement of our understanding of microbial ecology in a pond environment.

Keywords: bacterial diversity, 16S rRNA gene, mariculture ponds, diseased eels, phylogenetic analysis

Introduction

Microorganisms drive biogeochemical cycles in coastal ecosystems by fixing and remineralizing

nutrients and regulating inorganic nutrient and carbon fluxes (Moran, Gonzalez & Kiene 2003; Kirchman, Elifantz, Dittel, Malmstrom & Cottrell 2007). They have major roles in pond aquaculture, particularly with respect to productivity, nutrient cycling, the nutrition of the cultured animals, water quality, disease control and the environmental impact of the effluent (Moriarty 1997). As a food supplier to humans, marine aquaculture is one of the fastest-growing industries and large-scale coastal aquaculture has become an integrated part of coastal ecosystems around the world. It is known that mariculture practice has a profound impact on the biogeochemistry of the water column and sediment in mariculture zones (Qian, Wu & Ni 2001; Souchu, Vaquer, Colllos, Landrein & Deslous-Paoli 2001; La Rosa, Mirto, Favaloro, Savona, Sara, Danovaro & Mazzola 2002; Sara 2007). Maricultue practices disperse particulate organic matter into underlying sediments (Holmer & Kristensen 1992) and enrich the surrounding waters with nutrients (Loya, Lubinevsky, Rosenfeld & Kramarsky-Winter 2004). As a result, fish farms may stress nearby coastal ecosystems and lead to ecosystem degradation and decreased resilience (Hughes & Connell 1999).

Within the context of microbial contributions to maricultural ecosystems, a variety of interactions occur between specific microbial taxa and the surrounding environment or farmed fish. For example, *Cytophaga* bacteria in the phylum Bacteroidetes were proficient in utilizing high-molecular-weight dissolved organic matter (Cottrell & Kirchman 2000) and such DOC are abundant in mariculture ponds. Moreover, microbes may be the most sensitive component in ecosystems to environmental perturbations and their composition may serve as an indicator of ecosystem health or forecast for disease outbreak. A variety of *Vibrio* species in the sub-phylum Gammaproteobacteria have been found that may cause fish diseases, such as *V. alginolyticus* (Austin, Stobie, Robertson, Glass, Stark & Mudarris 1993), *V. anguillarum* (Qin, Wang, Zhang & Yang 2006) and *Vibrio cholerae* (Kiiyukia, Nakajima, Nakai, Muroga, Kawakami & Hashimoto 1992). Understanding the microbial composition in mariculture environments can provide an insight into large-scale ecological processes in a coastal environment and help assess the risk of exposure of farmed fish to pathogens.

Currently, studies on microorganisms in mariculture are mainly focused on bacterial physiological characteristics (Caruso, Genovese, Mancuso & Modica 2003), bacterial density (Pitta, Apostolaki, Tsagaraki, Tsapakis & Karakassis 2006), isolation of antibiotic-resistant bacteria (Dang, Zhang, Song, Chang & Yang 2006) and the usage of probiotics in mariculture (Qi, Zhang, Boon & Bossier 2009). Management of the activities of microorganisms in food webs and nutrient cycling in ponds is necessary for optimizing production (Moriarty 1997). However, the influence of fish farm on the natural microbial diversity has received little attention and our understanding of microbial communities in numerous kinds of mariculture ponds is limited.

In China, mariculture production reached a striking amount of 13 073 400 tonnes in 2007, 1.4% of which were lost owing to diseases and pollution (Chinese Agriculture Statistics 2006). Disease is the primary constraint to the aquaculture of aquatic species in the world (Bondad-Reantaso, Subasinghe, Arthur, Ogawa, Chinabut, Adlard, Tan & Shariff 2005). Fish diseases in China were mainly caused by bacterial pathogens, including *Edwardsiella tarda*, *Vibrio anguillarum*, *Vibrio harveyi*, *Photobacterium damselae* subsp. *Piscicida*, etc. (Qi *et al.* 2009). The widely distributed mariculture ponds in China coasts and the frequent outbreaks of fish diseases urge us to monitor microbial distribution and their changes to environmental perturbations for the purposes of decreasing fish diseases, improving farming efficiency and strengthening environmental management as well. In this study, we sampled a variety of coastal maricultural ponds farming grouper, prawn, abalone and algae in Southeast China and amplified 16S rRNA genes directly from community DNA to gain an insight into *in situ* bacterial diversity. Molecular methods including denaturing gradient gel electrophoresis (DGGE) and clone library as well as the traditional culturing method were applied with the aim of comparing the bacterial diversity in different maricultural ponds and revealing the difference between bacterial composition in diseased eels and that in a natural environment.

Materials and methods

Sample collection

Various ponds farming different commercial aquaculture types were sampled during 2004–2006 (Fig. 1, Table 1). These ponds represent typical coastal aquaculture environments in Southeast China and their farm history ranged from 3 to 5 years. *Epinephelus diacanthus* (ED, Grouper) pond, *Penaeus vannamei* (PV, Prawn) pond, *Haliotis diversicolor* supertexta

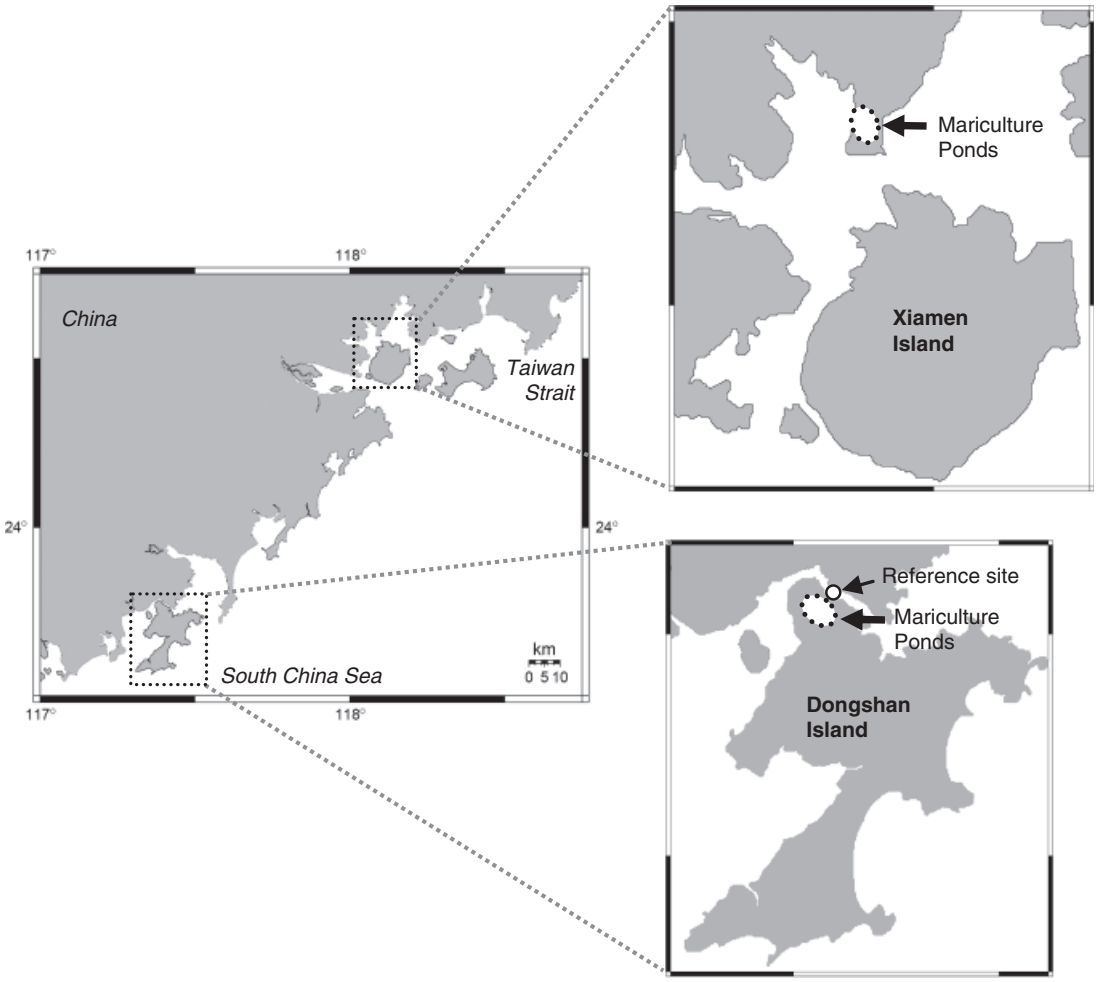

Figure 1 Sampling locations.

(HDS, Abalone) pond I and *Gracilaria verrucosa* (GV, Algae) pond are located in the Dongshan mariculturral zone (Fig. 1), north of the Dongshan Island in the southeast coastal region of China (23°45′N, 117°22′E). *Penacus orientalis* (PO, Prawn) pond and another HDS pond II are located north of the Xiamen Island (Fig. 1), north of the Dongshan Island with an approximately 150 km distance. Both of them are located in southwest of the Taiwan Strait. The dominant ocean current belongs to the Southeast coastal water system of China.

Each pond has an approximate length of 100 m, a width of 85 m and a depth of 1.5 m. Surface water was sampled in triplicate from an approximate depth of 30 cm at each pond and a non-mariculture reference site. Surface sediment and water samples were collected in polycarbonate bottles that were rinsed in acid once and distilled water three times, and autoclaved. Samples were returned immediately to the lab and kept in the dark until analysis. Diseased eels were collected for pathogen isolation from various eel ponds in Southeast China during 2002–2008. Pathogens were maintained at $-80\,^{\circ}C$ in the laboratory culture centre.

Extraction of community DNA

Approximately 1–3 L of water samples for DNA extraction were pre-filtered through a nylon mesh (100 μm pore size) to remove debris. Samples were subsequently filtered onto 0.22 μm pore-size nylon filters (Gelman Sciences, Ann Arbor, MI, USA). Filters were immediately frozen in liquid nitrogen and

Table 1 Information about the samples and mariculture ponds

Sample type		Farming period	Location	Sampling time	Sampling type	Analysis method
Epinephelus diacanthus pond	Grouper	Over 5 years	Dongshan Island, Southeast China	October 2004	Surface water	DGGE
Penaeus vannamei pond	Prawn	Over 5 years	Dongshan Island, Southeast China	October 2004	Surface water	DGGE
Haliotis diversicolor supertexta pond I	Abalone	Over 5 years	Dongshan Island, Southeast China	October 2004	Surface water	DGGE
Gracilaria verrucosa pond	Algae	Over 5 years	Dongshan Island, Southeast China	October 2004	Surface water	DGGE
Non-mariculture reference site	–	–	Dongshan Island, Southeast China	October 2004	Surface water	DGGE
Penacus orientalis pond	Prawn	Over 3 years	Xiamen Island, Southeast China	July 2005	Sediment	Clone library
Haliotis diversicolor supertexta pond II	Abalone	Over 5 years	Xiamen Island, Southeast China	November 2006	Surface water	Clone library, Culture
Diseased eels	Fish	30–90 days	Xiamen Island, Southeast China	2002–2008	Livers	Culture

DGGE, denaturing gradient gel electrophoresis.

stored in −80 °C. For DNA extraction, filters were cut into pieces by a sterilized scissor, placed into a 15 mL centrifugal tube, vortexed with 2.0 mL of lysis buffer (40 mM EDTA, 50 mM Tris-HCl, 0.75 M sucrose) and then incubated at −80 °C for 15 min. Tubes were transferred to 37 °C incubation for 6 min. Additional 2.0 mL of lysis buffer with lysozyme (1.0 mg mL^{-1}) was added. Samples were incubated at 37 °C for 30 min. Proteinase K (0.2 mg mL^{-1}) and 1% of sodium dodecyl sulphate (final concentration) were added, followed by incubation at 55 °C for 2 h. The lysate recovered by centrifugation was poured into a new 15-mL centrifugal tube, and 1.5 mL of 10.5 M ammonium acetate plus 10 mL of ice-cold 100% ethanol were added to precipitate the DNA at −20 °C for 2 h. The DNA pellets were rinsed once with phenol-chloroform-isoamyl alcohol (25:24:1), once with chloroform-isoamyl alcohol (24:1) and twice with pre-chilled 70% ethanol. DNA were dried under vacuum, gently suspended in 40 μL of TE buffer (pH 7.4) and stored at −20 °C. DNA quality was checked by electrophoresis on a 1% agarose gel. For sediment sample, 1 g soil was suspended with buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0). The subsequent treatments were generally the same as that for filter samples.

Isolation of bacterial cultures

One hundred and fifty millilitres of water of HDS pond II was plated onto solid 2216E media (Difco formula, Detroit, MI, USA). After 24 h of growth at 26 °C, single colonies were picked out and maintained at 4 °C. Pathogens were isolated from the internal organs of diseased eels by plating onto solid media. The pathogenicity of isolated pathogens was verified by artificial infections. DNA extraction of cultures refers to a standard CTAB protocol for bacteria (Sambrook 2001).

PCR amplification

Polymerase chain reaction amplifications of 16S rDNA gene fragments for DGGE were conducted in a T3 thermal cycler (Biometra, Gottingen, Germany), using the primers 341F-GC (5′-CGCCCGCCGCGC GCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGG GAGGCAGCAG-3′) and 518R (5′-ATTACCGCGG CTGCTGG-3′) (Yu & Morrison 2004). Polymerase chain reaction was performed under the following conditions: initial denaturation at 95 °C for 3 min, pause at 80 °C for hot start, followed by 20 touchdown cycles of denaturation at 94 °C for 1 min, annealing at 65 °C (with the temperature decreasing 0.5 °C each cycle) for 1 min and extension at 72 °C for 3 min, continued with six cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min and ending with a final extension at 72 °C for 7 min. For near full-length 16S rRNA gene amplification, universal bacterial primers 27F (5′-AGAGTTTGATCATGGCTC AG-3′) and 1492R (5′-GGTACCTTGTTACGACTT-3′) were used. The PCR mixture contained 30–50 ng of template DNA, 10 μL 10 × PCR buffer, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 200 μM of each dNTP and 4 U Taq polymerase (TaKaRa, Dalian, China) in a total volume of 100 μL. The reaction conditions were as follows: initial denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, with a final extension step at 72 °C for 10 min.

Amplification with each sample was performed in triplicate. Polymerase chain reaction products were purified using the Gel Extraction Kit (Sangon, Shanghai, China) according to the manufacturer's instructions. Ligation into the pMD18-T vector (TaKaRa) and transformation into *Escherichia coli* DH5α were performed using standard procedures. The ampicillin-resistant clones were randomly picked out and screened for target inserts by performing colony PCR. Positive clones were sequenced on an ABI 377A automated sequencer (Applied Biosystems, Foster City, CA, USA).

DGGE

Denaturing gradient gel electrophoresis was performed using a D-Code system (Bio-Rad, Hercules, CA, USA). Approximately 600 ng of purified PCR products were loaded onto an individual lane in the 8% polyacrylamide gel (ratio of acrylamide to bis acrylamide, 37.5:1) and submerged in 0.5 × TAE buffer (20 mM Tris, 20 mM acetic acid, 0.5 mM EDTA, pH 7.4). Denaturing gradients ranged from 40 to 60% (a 100% denaturant was defined as 7 M urea and 40% deionized formamide). Electrophoresis was performed at 60 V and maintained at 60 °C for 16 h. After electrophoresis, gels were stained for 50 min in Milli-Q water containing SYBR green I (1:10 000 dilution), destained for 15 min with Milli-Q water, visualized using the Dark Reader Transilluminator (Clare Chemical Research, Dolores, CO, USA) and photographed using the Bio-Rad Gel Doc XR system. The digital images were analysed using the QUANTITY ONE software (version 4.6.1) to obtain densitometric profiles. Denaturing gradient gel electrophoresis bands were sequenced after excision from the gel and reamplification. Briefly, the bands were excised, resuspended in 20 μL of MilliQ water and stored at 4 °C overnight. An aliquot of the supernatant was used for PCR reamplification with the original primer set. Between 30 and 50 ng of the reamplified PCR product was used for sequencing.

Sequence analysis

All sequences were imported into the ARB software package [version 4.32-MGG-5939M; O. Strunk & W. Ludwig, Technische Universität München (http://www.arb-home.de)]. The ARB database was released on 24 March 2009 with 868 390 aligned rRNA sequences. Sequence alignments and phylogenetic analysis were performed using the ARB program with regions of sequence ambiguity being excluded. Framework trees were calculated using fastDNAmL, a maximum-likelihood method implemented in ARB, with only almost-full-length sequences (≥1400 bases). Homologous nucleotide positions, based on the filter of the ARB database, were included in the alignment and used for the comparison analysis. Short DGGE sequences were added to the tree using the add-by-parsimony algorithm, which allows the addition of short sequences to existing phylogenetic trees without changing the global tree topologies. The CHECK-CHIMERA program of the Ribosomal Database Project server was used for searches of chimera artefacts (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU). All sequences obtained in this study were deposited in the GenBank database under the following accession numbers: DQ089483-DQ089518, FJ786105-FJ786141, FJ786085-FJ786104, EU651799-EU651812, FJ494884-FJ494907 and FJ628392-FJ628394.

Results

PCR-DGGE analysis of bacterial communities in the Dongshan Island

Samples from the Dongshan Island maricultural zones were collected in the same month. Denaturing gradient gel electrophoresis was used to perform a fast comparison of the surface bacterioplankton community structure in different sites. Despite geographically close locations of the sampling sites in the Dongshan Island, PCR-DGGE results show a significantly different pattern in their components (Fig. 2). The most abundant bands (20 DGGE bands) were retrieved from the GV pond sample, much higher than those from the non-mariculture reference site (12 DGGE bands). *Epinephelus diacanthus* pond, PV pond and HDS pond I samples show 18, 11 and 18 DGGE bands respectively. The bacterial diversity in mariculture ponds was generally increased compared with the reference site, except for the PV pond.

A total of 36 distinct DGGE bands were identified and sequenced. Among them, only two (band 2 in Bacteroidetes and band 15 in Alphaproteobacteria) were shared in all five samples and, in addition to these two common groups, only five (bands 13 and 30 in Alphaproteobacteira, bands 24 in Gammaproteobacteria, band 20 in Cyanobacteria and band 8 in Chloroplasts) appeared simultaneously in over three

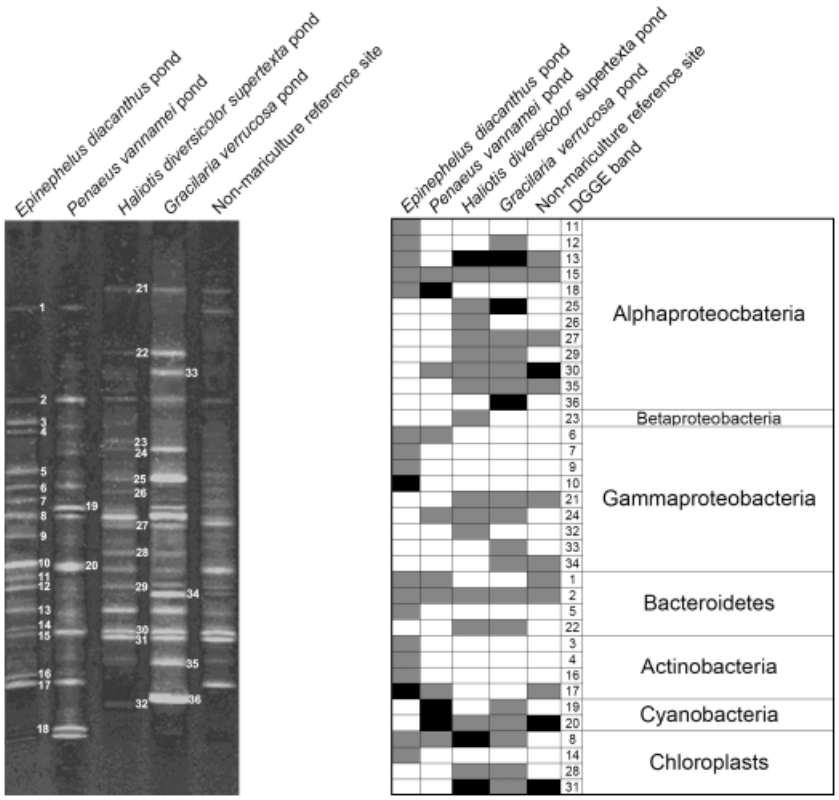

Figure 2 Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA genes in the Dongshan Island mariculture ponds. Left, DGGE gel marked with sequenced bands; Right, occurrence of each DGGE band in the samples with blank, grey and black squares representing absence, presence and dominance respectively.

mariculture ponds. Twenty-four bands were not detected in the non-mariculture reference sample. These groups much likely originated from the minor groups below the detection limit in non-mariculture seawaters because DGGE could only detect the dominant bacterial groups in a community. These results indicate that mariculture practice with different animals or plants may have a different influence on the bacterioplankton compositions.

Bacterial communities in the Dongshan samples were dominated by Alphaproteobacteria, followed by Gammaproteobacteria and Bacteroidetes. A few sequences from Cyanobacteria or Chloroplasts were present in all samples, suggesting that autotrophs remain an important fraction in mariculture environments where organic nutrients that favour heterotrophic bacteria were frequently overloaded. At the phylum or the subphylum level, there is no obvious difference among the samples investigated, except for Betaproteobacteria that only appeared in the HDS pond I. However, at the species level, the most abundant groups are different among four mariculture ponds. For example, the most abundant sequences in the ED pond were from two species affiliated with Gammaproteobacteria and Actinobacteria, respectively, whereas in the PV pond there was an Alphaproteobacteria and two *Cyanobacteria* species.

Clone library analysis of bacterial communities in the prawn pond sediment and abalone pond water

Thirty-seven sequences were obtained from the PO pond sediment clone library and were classified into Gammaproteobacteria (16, sequence number), Deltaproteobacteria (7), Alphaproteobacteria (6), Acidobacteria (3), Actinobacteria (2), Firmicutes (1), TM6 (1) and Chloroplast (1) (Fig. 3). Twenty sequences were identified from the bacterioplankton's clone library of the pond II farming abalone HDS and were classified into Gammaproteobacteria (11), Alphaproteobacteria (2) and Bacteroidetes (2) (Fig. 3). On the phylum or the subphylum level, no significant difference existed among the bacterial communities in mariculture pond surface water. However, the PO

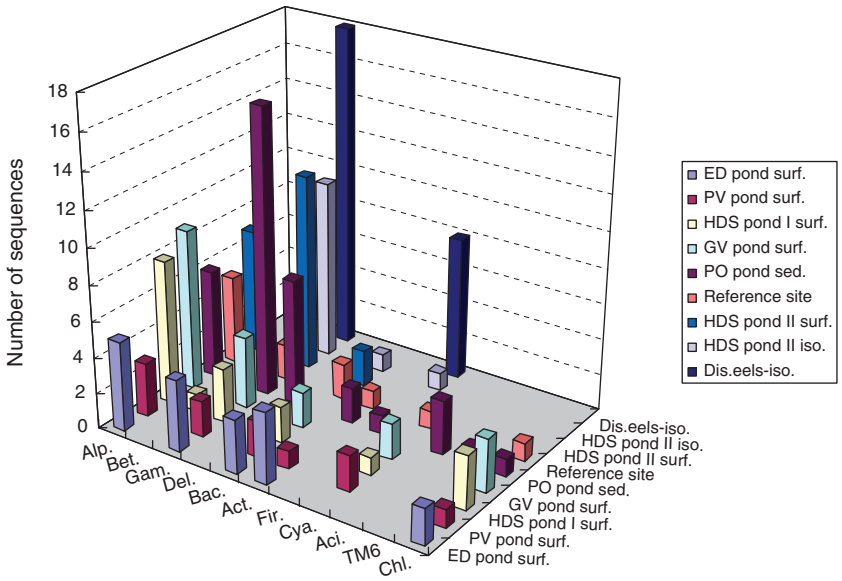

Figure 3 Abundance of sequences in each phylum or subphylum in different samples. ED pond surf., surface water of the *Epinephelus diacanthus* pond; PV pond surf., surface water of the *Penaeus vannamei* pond; HDS pond I surf., surface water of the *Haliotis diversicolor* supertexta pond I; GV pond surf., surface water of the *Gracilaria verrucosa* pond; PO pond sed., sediment of the *Penacus orientalis* pond; Reference site, the non-mariculture reference site in the Dongshan Island coast; HDS pond II surf., surface water of the *H. diversicolor* supertexta pond II; HDS pond II iso., bacterial cultures isolated from the water in the *H. diversicolor* supertexta pond II; Dis. eels-iso., bacterial cultures isolated from diseased eels.

pond sediment showed a much more complex bacterial distribution than all the surface waters in this study. Deltaproteobacteria, Firmicutes, Acidobacteria and TM6 occurred only in the sediment sample, indicating the distinct difference between surface water and sediment as habitats for bacteria.

Bacterial cultures from abalone pond water and diseased eels

Twelve dominant bacterial cultures were isolated from the water of the HDS pond II. They are identified as Gammaproteobacteria (10, number of species), Bacteroidetes (1) and Firmicutes (1) respectively (Fig. 3). In contrast to the abundant presence of Alphaproteobacteria in the HDS pond II surface water clone library, no member of this subphylum was cultured on 2216E media. Similarly, one Firmicutes species was identified only using the culture method. Gammaproteobacteria dominated in both HDS pond II clone library and cultures, suggesting the strong cultivability of this group. Similarly, Gammaproteobacteria dominated in cultures isolated from diseased eels, with a total of 18 species identified. The other group cultured from diseased eels was Firmicutes, with eight species identified. In contrast, no *Firmicutes* species was detected in the pond water samples investigated.

Phylogenetic analysis

A total of 131 sequences were obtained in this study and were subjected to phylogenetic analysis (Fig. 4). Sixty-four sequences (48.9%) were clustered into Gammaproteobacteria and formed the most abundant group, followed by Alphaproteobacteria (25, number of sequences; 19.1%), Firmicutes (10, 7.6%), Bacteroidetes (7, 5.3%), Deltaproteobacteria (7, 5.3%), Actinobacteria (6, 4.6%), Chloroplast (5, 3.8%), Acidobacteria (3, 2.3%), Cyanobacteria (2, 1.5%), Betaproteobacteria (1, 0.7%) and candidate phylum TM6 (1, 0.7%).

Gammaproteobacteria

Out of 64 Gammaproteobacteria sequences, only 18 were not clustered into the known family or genus and they were grouped into nine uncultured clades. The genus *Aeromonas* represents the largest clade in Gammaproteobacteria and is comprised of 13 sequences, all of which were cloned from diseased eels. The second most abundant is the *Vibrio* clade and it is comprised of nine sequences, which originated from

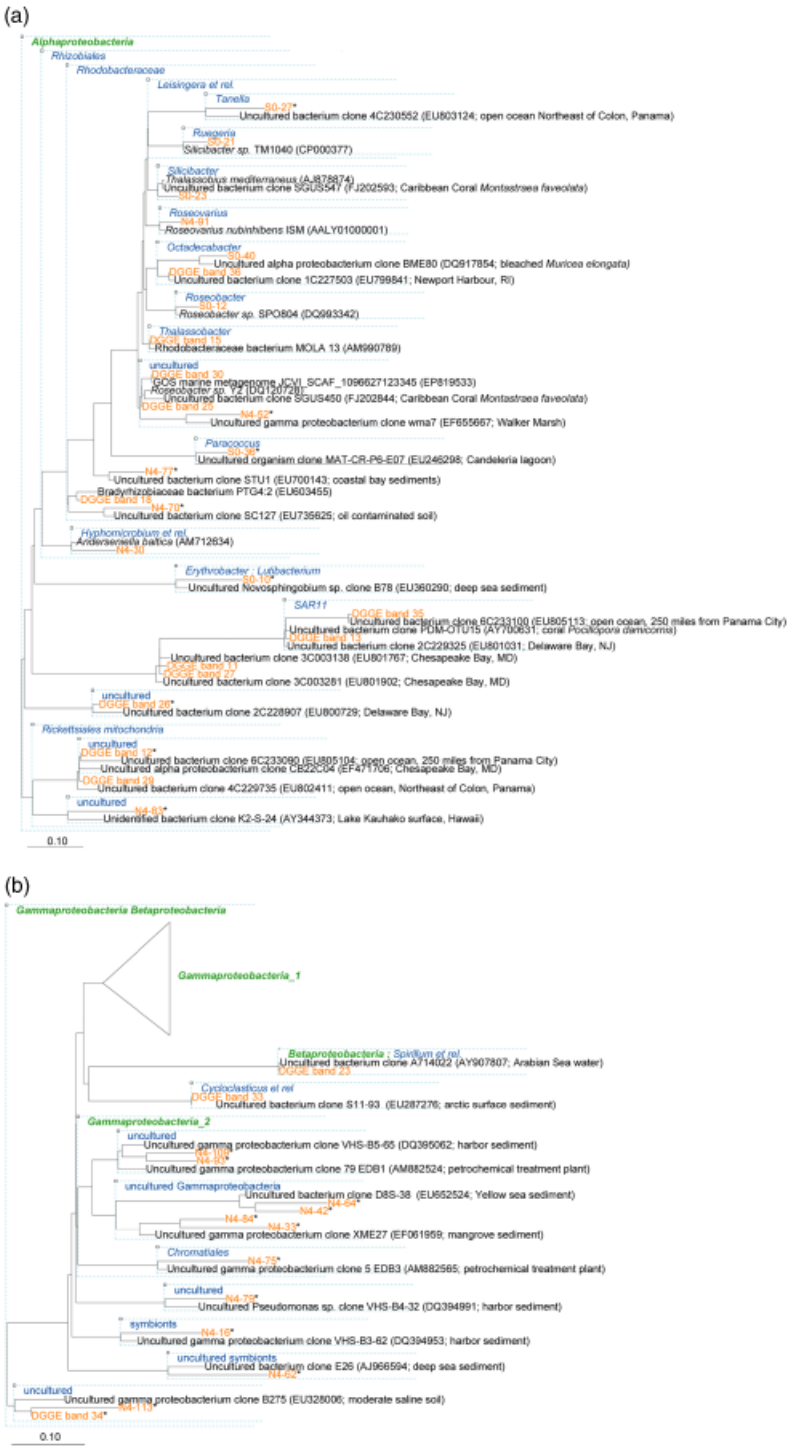

Figure 4 Phylogenetic analysis of 16S rRNA gene sequences cloned from mariculture ponds and diseased eels. (a) Alphaproteobacteria; (b), (c) Gammaproteobacteria; (d) Deltaproteobacteria; (e) Firmicutes and Actinobacteria; (f) and other groups including Chloroplasts, Cyanobacteria, Bacteroidetes, Acidobacteria and candidate phylum TM6. Trees were constructed using the ARB program. Sequences that show $<95\%$ similarity to their nearest neighbours are marked with an asterisk. Cluster names are defined by the ARB classification system based on the aligned 16S rRNA gene sequences. The source environment of each uncultured neighbour is shown in the parentheses.

Figure 4 Continued.

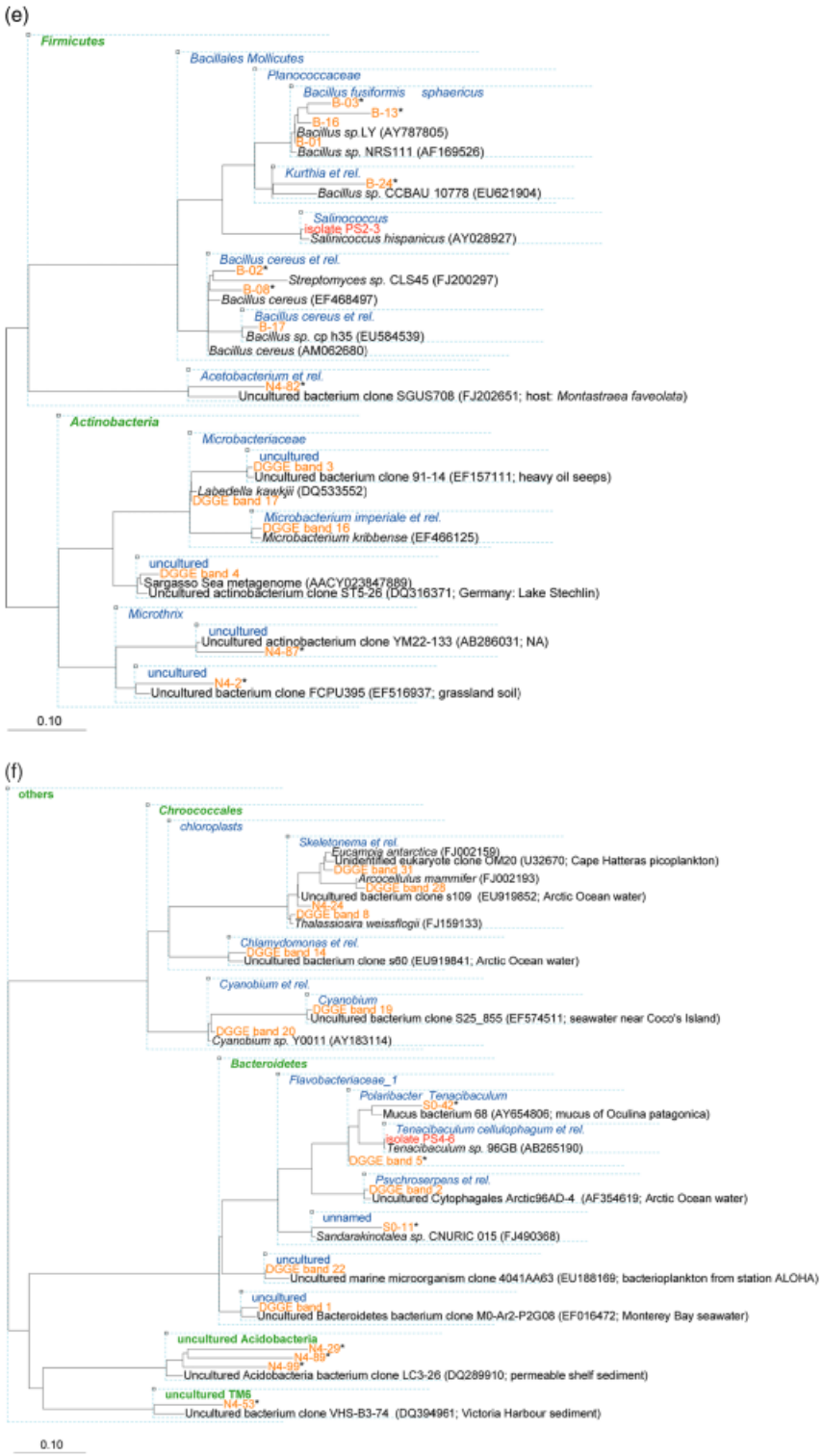

Figure 4 Continued.

the water in HDS pond II. The clades *Alteromonadales* and *Escherichia* contain six and five sequences respectively. Most *Escherichia* species (four out of five) were cloned from diseased eels, and the genus *Pseudoalteromonas* in *Alteromonadales* is comprised of four isolates from HDS pond II. Another dominant clade in Gammaproteobacteria is *Oceanospirillales*, which contains five sequences from water or sediment environmental clones. The least abundant family or genus are *Halomonadaceae* (three sequences), *Marinomonas* (3) and *Cycloclasticus* (1). The uncultured clades are constituted mainly by prawn sediment clones. Half (32) of the sequences in Gammaproteobacteria are phylogenetically closely related to the identified or the cultured species. The majority of the closest uncultured neighbours of Gammaproteobacteria sequences are of soil or sediment origin (Table 2). Five, four and three neighbours are of marine animal/plant, coast/estuary and freshwater origin respectively (Table 2).

Alphaproteobacteria

About half of the Alphaproteobacteria sequences (13 out of 25) are classified into the known genera, including *Tanella*, *Ruegeria*, *Silicibacter*, *Roseovarius*, *Octadecabacter*, *Roseobacter*, *Thalassobacter*, *Paracoccus*, *Hyphomicrobium*, *Erythrobacter* and SAR11. Nine out of the twelve sequences that cannot be classified into a known genus are affiliated with the order *Rickettsiales* and the family *Rhodobacteraceae*. Only six Alphaproteobacteria sequences (S0-21, S0-12, N4-91, N4-30 and DGGE bands 15 and 18) are phylogenetically closely related to the identified or the cultured species. Most of the closest uncultured neighbours of Alphaproteobacteria sequences are of open sea or coast/estuary origin (Table 2). Neighbours of marine animal/plant, soil/sediment, lagoon/marsh and freshwater origins were also found (Table 2), which suggests that Alphaproteobacteria in a mariculture environment have an extensive variety of sources.

Firmicutes and Actinobacteria

All sequences, except N4-82 in Firmicutes, were cloned from cultures, with eight from diseased eels and one from prawn sediment. All of them were classified into known genera, including *Bacillus* (7), *Kurthia*, *Salinococcus* (1) and *Acetobacterium* (1). Only one Firmicutes sequence (N4-82, prawn sediment) was phylogenetically closely clustered with an uncultured clone, which originated from a parasitic bacterium in coral *Montastraea faveolata*. The six sequences (four from surface water and two from sediment) in Actinobacteria were classified into three genera, *Labedella*, *Microbacterium* and *Microthrix*, and two uncultured clades. The closest uncultured neighbours of Actinobacteria sequences are primarily of soil/sediment and open sea origin (Table 2).

Deltaproteobacteria

All seven Deltaproteobacteria sequences were cloned from prawn sediment. N4-54 and N4-25 were classified into the genera *Desulfosarcina* and *Desulfobacterium* respectively. Another five sequences were grouped into three uncultured clades. All of their closest neighbours were cloned from marine sediment, in accordance with their source (Table 2).

Other groups

Seven sequences (six from water and one from sediment) were classified into photosynthetic autotrophs

Table 2 Statistical analysis of the source environment of the closest uncultured neighbours of the sequences obtained in this study

Source environment of the closest uncultured neighbours	Number of the closest neighbours											
	Alp.	Bet.	Gam.	Del.	Bac.	Act.	Fir.	TM6	∑	Water*	Sediment*	tissue*
Open sea	5	1			2	1			10	8		
Coast/estuary	5		4		1				10	11		
Freshwater	1		3						4	1	3	
Marine animal/plant	3		5		1		1		10	6	2	1
Lagoon/marsh	2								2	1	1	
Soil/sediment	3		14	5		2		1	25	5	19	3
NA						1			1		1	

Sequences are grouped by their affiliations and source environment.
*Sequences of this study were grouped by their source environment.
Alp., Alphaproteobacteria; Bet., Betaproteobacteria; Gam., Gammaproteobacteria; Del., Deltaproteobacteria; Bac., Bacteroidetes; Act., Actinobacteria; Fir., Firmicutes.

clades, including cyanobacteria and eukaryotic chroloplasts. Three autotrophic genus were identified, i.e. *Skeletonema*, *Chlamydomonas* and *Cyanobium*. Among Bacteroidetes, seven sequences were included and clustered into two known genera, *Plaribacter/Tenacibaculum* and *Psychroserpens*, and three uncultured clades. The closest uncultured neighbours of Bacteroidetes sequences are of open sea, coast/estuary or marine animal/plant origin (Table 2). Acidobacteria and TM6 clade contain three and one sequence, respectively, all of which were cloned from the prawn sediment sample.

In total, 62 clusters were identified in the 131 sequences obtained in this study by phylogenetic analysis, out of which 34 clusters belong to known genera. For the non-photoautotrophic bacteria, soil/sediment was the major source environment of their closest neighbours, followed by open sea, coast/estuary and marine animal/plant sources (Table 2).

Discussion

Thousands of mariculture ponds are distributed throughout the coast of Southeast China. The Dongshan Island and Xiamen Island are among the excessively exploited regions. Severe environmental issues have emerged there in the past decade, such as pollution caused by fish farm effluents, eutrophication, reduced water quality, disease prevalence, antibiotics abuse, etc. These problems are directly or indirectly connected to microbes or microbial functions in biogeochemical cycling. However, microbial communities in these regions are largely unknown. By sampling in a variety of ponds farming different animals or plants around the Dongshan Island and Xiamen Island and in the tissues of diseased eels, we observed a dramatic difference in bacterial diversity across the sites studied.

Huge differences in the bacterial composition in adjacent mariculture ponds farming different animals or plants

Profiles from DGGE revealed substantial differences in the bacterial community composition in aquaculture ponds that are adjacent to each other in the Dongshan Island. The dominant bacterial groups (Alphaproteobacteria, Gammaproteobacteria, Bacteroidetes and Actinobacteria) were generally consistent across all water samples including that from the Xiamen Island. However, in comparison with the non-mariculture reference site in the Dongshan Island at the species level, a total of 24 novel species appeared in maricultured seawater. One possibility is that the abundance of these species in non-maricultured seawater is below the detection limit by PCR-DGGE and when transferred into mariculture environments, they may outcompete other bacteria in the changed physical and chemical conditions. The other possibility is that they are originally associated with aquacultured animals or feed and are released into mariculture ponds.

It should be noticed that no species in the reference site disappeared entirely in all ponds. The ED pond underwent the heaviest losses in bacterial species of the reference site, with seven species disappearing, but another 13 new species appeared there. The least losses occurred in the GV pond, where only two species disappeared. These data imply that different mariculture practices exert different selective pressures on their associated microbial community and microbial species in natural seawater has the potential to adapt to aquaculture conditions. Only two species (represented by band 15 in Alphaproteobacteria and band 2 in Bacteroidetes) were shared in all five samples. Sequencing results show that they are classified into the genera *Thalassobacter* and *Psychroserpens* respectively.

Thalassobacter represents a newly identified genus of aerobic anoxygenic phototrophic bacteria (AAPB) (Macian, Arahal, Garay, Ludwig, Schleifer & Pujalte 2005) that are gram-negative, strictly aerobic, chemoorganotrophic, slightly halophilic and produce bacteriochlorophyll *a*. AAPB occupy a unique position in marine ecosystems due to their special physiology of having a photosynthetic and heterotrophic life style (Yurkov & Beatty 1998) and has been reported to distribute in global oceans (Jiao, Zhang, Zeng, Hong, Liu, Chen & Wang 2007; Yutin, Suzuki, Teeling, Weber, Venter, Rusch & Beja 2007) including coastal regions (Du, Jiao, Hu & Zeng 2006; Waidner & Kirchman 2007). However, to our knowledge, there was no previous report indicating that AAPB was distributed in mariculture environments. An AAPB-affiliated 16S rRNA gene sequence was retrieved from all the ponds we investigated, combined with the fact that two clones (S0-12 and 10 from the HDS pond II) were classified into another two AAPB genera, *Roseobacter* and *Erythrobacter*, respectively, suggesting that mariculture pond may be another undiscovered habitat for marine AAPB; nevertheless, direct evidences by isolating AAPB cultures or detecting *in situ* bacteriochlorophyll *a* signals are needed to support this speculation.

The genus *Psychroserpens* of Bacteroidetes is defined based on a type strain *Psychrosepens burtonensis* that is a psychrophilic bacterium isolated from Antarctic lacustrine and sea ice habitats (Bowman, McCammon, Brown, Nichols & McMeekin 1997). The fact that a *Psychrosepens*-affiliated sequence was obtained from a subtropical Chinese mariculture pond suggests that this exclusively psychrophilic genus has close mesophilic relatives in subtropic seas. Generally, members of Bacteroidetes are known to play an important role in the degradation and consumption of aquatic organic material (Cottrell & Kirchman 2000). Additionally, fish gut microflora was often dominated by Bacteroidetes (Kim, Brunt & Austin 2007). It is reasonable to consistently find Bacteroidetes species across all mariculture ponds due to the high input of organic nutrients by feeding and fish faeces in them.

In addition to DGGE, multiple approaches including clone library construction and culturing on plates were used in this study. The 16S rRNA clone libraries provided a survey of the microbial community composition, although comparisons among communities may be limited by low numbers of sequenced clones from each library. Clone libraries allow sequencing of near-full-length 16S rRNA genes and thus provide more information for phylogenetic analysis compared with DGGE in which usually sequences with a length of < 300 bp are acquired. Analyses of bacterioplankton in the abalone HDS ponds I and II by DGGE and the clone library, respectively, show that the bacterial diversity between both ponds has few overlaps. For example, the genera *Tanella*, *Ruegeria*, *Silicibacter*, *Paracoccus* and *Erythrobacter* in Alphaproteobacteria were only detected by clone library analysis; all sequences in the *Vibrio* cluster in Gammaproteobacteria were obtained by clone library and culturing methods. The contrasting results may be partly due to the inherent differences between bacterial communities in both ponds, but they also suggest that the clone library method is a good complement to DGGE in bacterial diversity analysis and simultaneous application of both approaches is necessary for revealing more diversities in complex samples.

Highly diverse bacterial communities and their possible controlling factors in mariculture ponds

Phylogenetic analysis of the sequences obtained by either DGGE or clone library or culturing shows that mariculture ponds harbour highly diverse bacterial communities. The factors that determine the bacterial composition in aquaculture are complex and likely to include cultured animal species, culture methods, feed composition, faecal excretion habits and feeding techniques. Previous studies have demonstrated that different cultured animals in mariculture have a significant impact on aquatic environments (Qian *et al.* 2001; La Rosa *et al.* 2002; Vezzulli, Chelossi, Riccardi & Fabiano 2002). A comparative study of fish farming and non-farming environments demonstrated that the bacteria in fish-farm sediments were predominantly Gram-negative, while the bacteria in the non-farming reference site of the same study were largely Gram-positive (Vezzulli *et al.* 2002). It is known that there are specific bacterial populations associated with cultured shrimps (Lau, Jumars & Armbrust 2002), scallop larvae (Sandaa, Magnesen, Torkildsen & Bergh 2003), halibut (Jensen, Ovreas, Bergh & Torsvik 2004) and sponges (Mohamed, Rao, Hamann, Kelly & Hill 2008). These cohabitants may transfer into a free-living life style and thus change the composition of the bacterial communities in the water column.

Another factor of structuring bacterial communities in Chinese mariculture ponds may be the wide usage of antibiotics. In the farm history of the ponds we investigated, the usage of streptomycin (final concentration 2 ppm) was ever recorded. Antibiotics may have a major impact on the microbes and might select against some specific bacteria. It was reported that large quantities of bacteria resistant to antibiotics were present in maricultures (Smith, Hiney & Samuelsen 1994), and marine *vibrio* spp. may be the most predominant resistant isolates to antibiotics in some mariculture farms (Dang *et al.* 2006). After acclimation to aquaculture environmental conditions, the growth of some bacteria may be favoured, resulting in these groups becoming major components of the microbial communities. The composition and history of sediment may also have a strong influence on the microbe components in the upper water columns as nutrient and microbial cells in the sediment could be directly released into the waters.

Among the diverse bacterial groups, pathogen-related clusters may attract the most attention among fish farmers and the public. We observed the distribution of four major clusters with pathogenic potentials, i.e. *Escherichia*, *Vibrio* and *Aeromonas* in Gammaproteobacteria and *Bacillus* in Firmicutes. All sequences in the *Escherichia*, *Aeromonas* and *Bacillus* clusters were cloned from diseased eels, while all *Vibrio* spp. originated from water samples.

Although it is not possible to absolutely prove that these cultured and uncultured data represent pathogens, the combined data are indicative of unhealthy waters that can lead to rapid pathogen proliferation, such as *V. cholerae* (Mourino-Perez, Worden & Azam 2003; Worden, Seidel, Smriga, Wick, Malfatti, Bartlett & Azam 2006). Inhibiting the growth of these potential pathogens and maintaining them in low abundance in the waters surrounding farmed fish are of paramount importance to healthy mariculture.

Another notable finding of this study is that abundant putative novel species exist in mariculture ponds. The percentage of novel sequences (< 95% similar to any accession entry in the NCBI nucleotide database) is 44.3% (58/131) and, especially, all sequences in the Delataproteobacteria, Acidobacteria and the candidate phylum TM6 are novel, indicating that mariculture pond harbours a wide array of undescribed phylotypes. In comparison with the water column in open oceans from which many cultured bacteria have been described, mariculture ponds remain undersampled to a huge degree.

The advances in microbial ecology in mariculture ponds are attributed to the fact that our ability to understand microbes in natural environments has increased considerably with the development of molecular approaches that provide a far more accurate picture of community composition and activities. A loss in bacterial diversity might have adverse consequences for marine animal health and long-term observations of microbial diversity in mariculture ponds may be helpful for the prevention and control of fish diseases when we correlate diversity data with changes in environmental variables, the epidemiology of specific pathogens and the health status of species farmed.

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