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ORIGINAL ARTICLE

Diversity of purple nonsulfur bacteria in shrimp ponds with varying mercury levels

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Abstract This research aimed to study the diversity of purple nonsulfur bacteria (PNSB) and to investigate the effect of Hg concentrations in shrimp ponds on PNSB diversity. Amplification of the *pufM* gene was detected in 13 and 10 samples of water and sediment collected from 16 shrimp ponds in Southern Thailand. In addition to PNSB, other anoxygenic phototrophic bacteria (APB) were also observed; purple sulfur bacteria (PSB) and aerobic anoxygenic phototrophic bacteria (AAPB) although most of them could not be identified. Among identified groups; AAPB, PSB and PNSB in the samples of water and sediment were 25.71, 11.43 and 8.57%; and 27.78, 11.11 and 22.22%, respectively. In both sample types, *Roseobacter denitrificans* (AAPB) was the most dominant species followed by *Halorhodospira halophila* (PSB). In addition two genera, observed most frequently in the sediment samples were a group of PNSB (*Rhodovulum kholense*, *Rhodospirillum centenum* and *Rhodobium marinum*). The UPGMA dendrograms showed 7 and 6

Abbreviations: APB, anoxygenic phototrophic bacteria; AAPB, aerobic anoxygenic phototrophic bacteria; PNSB, purple nonsulfur bacteria; PSB, purple sulfur bacteria

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clustered groups in the water and sediment samples, respectively. There was no relationship between the clustered groups and the total Hg (Hg_T) concentrations in the water and sediment samples used (< 0.002–0.03 μg/L and 35.40–391.60 μg/kg dry weight) for studying the biodiversity. It can be concluded that there was no effect of the various Hg levels on the diversity of detected APB species; particularly the PNSB in the shrimp ponds.

1. Introduction

Shrimp farming is one of the most important aquacultural activities in Asia and South America, but farmers face many serious problems like shrimp diseases partly due to problems with the shrimp having to cope with poor water quality during cultivation (Rattanachuay et al., 2011). Only 20–30% of the nitrogen supplied from their feed is converted into shrimp biomass, while the rest accumulates at the bottom of the shrimp ponds (Kutako et al., 2009) and is converted anaerobically by microbes with the production of ammonia and also hydrogen sulfide (H_2S). Both these compounds are toxic for shrimp (Rao et al., 2000; Antony and Philip, 2006); this means that they should be controlled at levels that are safe for shrimp growth. Among the microbes associated with the carbon, nitrogen and sulfur cycles, of shrimp ponds, purple nonsulfur bacteria (PNSB) are probably the most useful to improve the water quality during shrimp cultivation (Antony and Philip, 2006; Zhou et al., 2009). PNSB are versatile organisms able to grow with photoautotrophic or photoheterotrophic or heterotrophic conditions depending on the availability of light, oxygen and a suitable source of carbon. This means that they can consume organic matter under light and dark conditions and some can also remove H_2S (Antony and Philip, 2006; Kornochalert et al., 2014).

Normally, PNSB exist in illuminated anoxic habitats in nature such as in aquatic sediments, of freshwater rivers and lakes, and wastewater treatment systems including shrimp ponds (Panwichian et al., 2010). PNSB are also considered to be beneficial microbes as their cells have a high protein content, they produce essential amino acids, and contain a high content of vitamin B12, ubiquinone and carotenoids (Shapawi et al., 2012; Kornochalert et al., 2014), so they have a great potential for use as probiotics in aquaculture (Shapawi et al., 2012). However, very little work has been published on the diversity of PNSB in shrimp ponds; hence it is worthwhile to study their diversity in shrimp ponds in order to gain knowledge that might be useful for finding a use for them during shrimp cultivation.

The sequencing of the *pufM* gene that codes for the M subunit of a pigment-binding protein in the photosynthetic reaction center has been used to study the diversity and phylogenetic composition of anoxygenic phototrophic bacteria (APB) such as the purple sulfur bacteria (PSB, *γ-Proteobacteria*), PNSB (*α-*, *β-Proteobacteria*) and aerobic anoxygenic phototrophic bacteria (AAPB) (*α-Proteobacteria*) (Yutin et al., 2007; Asao et al., 2011). This is because this gene is part of the unique *puf* operon that codes for the subunits of the light-harvesting complex (*pufB* and *pufA*), and the reaction center complex (*pufL* and *pufM*) (Beja et al., 2002) present in all purple bacteria.

It is well recognized that shrimp cultivation in Thailand has occurred over at least the last 30 years (Lebel et al., 2010), and shrimp ponds are generally located near coastal areas of the Thai peninsular because natural seawater is required for shrimp cultivation. Use of untreated seawater for shrimp cultivation may cause problems if the seawater is contaminated especially with heavy metals including mercury (Hg) and other pollutants (Thongra-ar and Parkpian, 2002; Cheevaporn and Menasveta, 2003; Panwichian et al., 2010). In some cases the pollutants have accumulated in situations where the shrimp ponds have been used over a long period of time. In addition Hg contamination in the shrimp ponds has been caused by impurities in agricultural fertilizers, lime and chemicals used during shrimp cultivation (Lacerda et al., 2011). The contamination of heavy metals; particularly Hg should be of concern because it is known to have a major effect on the activities of microbes and the biogeochemical processes which they mediate (Harris-Hellal et al., 2009). Hence, Hg contamination in long-term cultivated shrimp ponds could affect the dynamics and the diversity of bacterial communities during shrimp cultivation, in particularly PNSB. As far as we know this is the first report on the effects of Hg concentrations on PNSB in shrimp ponds. With regard to the above information, this work aimed to study the diversity of PNSB in shrimp ponds using the specific *pufM* gene for understanding which species of PNSB were dominant. This might help us to understand their physicochemical properties and roles and propose ways for them to be used optimally to facilitate the cultivation of shrimp in shrimp ponds. This also includes the influence of Hg concentrations on PNSB diversity and their ability to facilitate shrimp growth in the presence of Hg.

2. Materials and methods

2.1. Study areas and samples collecting in shrimp ponds

Water and sediment samples were collected from 16 shrimp ponds used for cultivating white shrimp (*Litopenaeus vannamei*) with different lengths of operation times in the coastal areas of the Thai Peninsular (Gulf of Thailand and Andaman sea). These shrimp ponds were in the districts of Ranot (RN: RN1 and RN2), Tepa (TP: TP1, TP2 and TP3) and Sating Phra (ST: ST1, ST2 and ST3), in Songkhla Province; the districts of Pak Panang (PN: PN1, PN2, PN3 and PN4) in Nakhon Si Thammarat Province; Kantang (KT: KT2), Sikao (SK) and Yan Ta Khao (YT) in Trang Province and Mueang (PT) in Pattani Province. Therefore, the 16 shrimp ponds were in eight districts; and all those in the same district were cultivated by the same owner. Hence, the operation and management systems for shrimp cultivation in the same district were not different. During intensive shrimp cultivation,

nutritionally complete (artificial diet) and mechanical aeration were used to feed shrimp and increase dissolved oxygen in all 16 shrimp ponds; while stocking density, but the feeding regime and supplementary diet feeding including other aquaculture practices depended on the farm owners. Composite subsamples were collected from each pond at various positions to obtain a representative sample of the pond by following two diagonals and a half point from each bank of the pond. Approximately 100 ml of water samples at 50 cm from the surface and 100 g of sediment samples from a depth of 5 cm were collected from each position. All the water and sediment samples were collected during shrimp cultivation near to shrimp harvesting that had shown no signs of shrimp weakness to study the PNSB diversity for evaluating any effects of Hg concentrations on PNSB diversity during the whole process of one crop cycle. As all collected samples were contaminated with Hg at various concentrations; thereby there was no control (without Hg contamination) for comparing PNSB diversity in its absence. Both sample types were kept in sterilized glass bottles and stored in an icebox during transportation, then maintained at 4 °C before further studies. In addition a portion of the water that was separated for determining the amount of total mercury (Hg_T) was preserved by adding 5 ml/L of 0.2 N bromine monochloride (BrCl) before storage.

2.2. DNA extraction

Water samples were centrifuged at 8000×*g* for 20 min to obtain the biomass pellets for DNA extraction following the method of Zhou et al. (1996). Then the biomass pellet and sediment samples were separately washed twice with Tris–EDTA (TE) buffer to eliminate inhibitors that could affect DNA extraction. Genomic DNA was extracted from each sample using 600 µl of lysis buffer (100 mM Tris–HCl pH 8.0, 100 mM sodium EDTA, phosphate buffer pH 8.0, 1.5 M NaCl, 1% CTAB), 10 µl lysozyme and 10 µl protinase K, followed by mixing and incubating at 37 °C for 30 min. After that 100 µl of 20% SDS was added into the mixture and further incubated at 65 °C for 30 min. The supernatant was collected after centrifugation at 6000×*g* for 5 min and mixed with an equal volume of chloroform: isoamyl alcohol (24: 1 v/v) then further centrifuged at 12,000×*g* for 5 min. The aqueous phase was recovered, mixed with 0.6 volumes of isopropanol and kept at −20 °C for 1 h to precipitate genomic DNA. The genomic DNA was obtained by centrifugation at 12,000×*g* for 10 min and rinsed with absolute ethanol, air dried, and dissolved in nucleotide-free dH_2O. Genomic DNA was purified with the Gel/PCR DNA fragment extraction kit (Geneaid, Taiwan) according to the manufacturer's instructions and left at −20 °C for any further steps.

2.3. Nested polymerase chain reaction (PCR)

Genomic DNA from each sample was amplified by nested PCR using the *pufL* and *pufM* primers. In the first round, the *pufLM* gene (1500 bp) was amplified by *pufL* forward: 5′-CTKTTCGACTTCTGGGTSGG-3′ (Zeng et al., 2009) and *pufM* reverse 750: 5′-CCATSGTCCAGCGCCAGAA-3′ (Achenbach et al., 2001). Each PCR reaction was amplified with 25 µl of PCR reaction mix (QIAGEN PCR Kit) containing 2.5 µl of 10× buffer with 1.5 mM $MgCl_2$, 2.5 µl of Q-Solution, 0.5 µl of 10 mM dNTP mix, 1.0 µl of 10 nM forward and reverse primer, 1.0 µl of DNA template, 0.1 µl of 5 U/µl *Taq* DNA polymerase and 16.4 µl RNase-free water. The reaction cycles were performed in a Thermal Cycle as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 50 s, 60 °C for 30 s, and 72 °C for 2 min, with a final extension step at 72 °C for 10 min. The PCR product from the first round was then used as the DNA template for the second round to amplify the *pufM* gene (233 bp) in the same way using the *pufM* forward 557: 5′-CGCACCTGGACTGGAC-3′-GC (Achenbach et al., 2001) and *pufM* reverse 750: 5′-CCATSG TCCAGCGCCAGAA-3′. The PCR mixture and reaction cycles for amplification followed the same procedure as for the first round with the exception of the extension time that was reduced to 30 s. The PCR products were separated by electrophoresis on a 0.8% agarose gel, then stained with ethidium bromide and the bands were observed on a UV transilluminator.

2.4. DGGE and phylogeny

The 233 bp-long *pufM* fragments from the water and sediment samples were separated in a 45–70% and 40–60% (w/v) denaturing gradient, respectively into a 0.8% (w/v) polyacrylamide gel. The electrophoresis was performed at 80 V, and 60 °C for 16 h. After electrophoresis, the polyacrylamide gel was stained using the SYBR Gold nucleic acid stain for 20 min. The images were detected and captured on a UV transilluminator. The *pufM* fragments were cut from the gel using Gel Cutting Tips (Cleaver Scientific, England) and re-amplified with the *pufM* forward and *pufM*750-AT-M13 primers. The PCR products were purified using the Gel/PCR DNA fragment extraction kit (Geneaid, Taiwan) according to the manufacturer's instructions and then sequenced with *pufM*750-AT-M13 as sequencing primers using the 1st BASE Laboratories Sdn Bhd (Malaysia) and were compared with the GenBank database in the NCBI website. Phylogenetic analysis was constructed by neighbor-joining using the free software MEGA 5.

2.5. Hg analysis

Water and sediment samples collected from each shrimp pond were used to determine the amount of total mercury (Hg_T). All tubes and bottles used for analysis were first cleaned by heating to 65 °C in 4 N HCl for 12 h, cooled and rinsed three times with deionized water, and then dried in a clean oven at 60 °C overnight. Method 1631 (2001) and method 1631 (2002) of the U.S. Environmental Protection Agency (USEPA) were used to prepare the sediment and water samples, respectively for detecting Hg_T and then analyzed by the Perkin-Elmer Flow Injection Mercury System 400 using $NaBH_4$ as the reducing reagent and 3% (v/v) HCl for the carrier solution.

2.6. Statistical analysis

All experiments in this study were conducted in three replicates. Mean values and the standard deviations are presented. Analysis of the data using one way ANOVA and significant differences among means were analyzed using the Duncan's multiple range test at a p-value < 0.05. DGGE profiles were

compared using the Jaccard coefficient based on the presence or absence of bands. Dendrograms were generated from the Jaccard coefficient using an unweighted paired group analysis of means (UPGMA) and the free trial XLSTAT.

3. Results

3.1. Diversity of purple nonsulfur bacteria in shrimp ponds

As stated in the introduction, the *pufM* gene that we used to study the diversity of PNSB in water and sediment samples collected from the sixteen shrimp pond sites was also present in other groups of APB so it was not specific for PNSB. Using the *pufM* gene primers, bands were detected after DGGE in 13 water samples (Fig. 1A) and 10 sediment samples (Fig. 1B). As some of the samples were detected in the different gels, therefore the same numbers of DGGE bands as shown in Fig. 1 were compared from the positions on the gels and also were confirmed from the results of sequencing analysis. The DGGE profiles from each site differed with many more bands appearing for each of the water samples compared to those from the sediment samples. However, from the water samples, bands 1 and 7 appeared in all while bands 5 and 6 were found from most sampling sites (Table 1). Moreover, there were some bands such as bands; 3, 8, 10, 11, 14, 16 and 21 that appeared frequently among the different sites. Some 35 of the more predominant bands from the water samples were subjected to DNA analysis. Only 3 were identified as known PNSB, 4 were identified as being PSB, 9 were derived from known AAPB and the remaining 19 were not recognized and therefore assigned as uncultured. It is possible that some of these could have been related to PNSB. As for the samples from the sediment only 18 bands were characterized, 4 were PNSB, 2 were PSB, 5 were AAPB while 7 were unrecognized (Table 2). Bands 1 and 7 were closely related to *Roseobacter denitrificans* OCh114 (AAPB) and *Roseibacterium elongatum* OCh323 (AAPB), and they were the dominant species in all the water samples while bands 5 and 6 that were closely related to *Halorhodospira halophila* H (PSB) and *Marivita* sp. RCC1921 (AAPB), respectively were found in most water samples.

In contrast, the DGGE profiles in the sediment samples (Fig. 1B) were quite different and had fewer DGGE profiles than in the water samples. However, lists of bacterial species in the sediment samples belonged to groups of PSB, PNSB and AAPB as well, and again band 1 was closely related to *R. denitrificans* OCh114 that was also the dominant species in the sediment samples followed by band 5, related to *H. halophila* H. For the PNSB group bands; 3, 17 and 18 closely corresponded to *Rhodovulum kholense* type strain JA297T, *Rhodospirillum centenum* SW and *Rhodobium marinum* JA211 (Table 2). The following species were detected in both sample types; *R. denitrificans* OCh114, *Marivita* sp. RCC1921, *Loktanella* sp. NP29 (AAPB), *H. halophila* H, *R. marinum* JA211 (PNSB, *α-Proteobacteria*) and *Rubrivivax gelatinosus* (PNSB, *β-Proteobacteria*). However, roughly 54.3% and 38.9% of the DGGE fragments from water and sediment samples respectively had no identified analog in the data base (Tables 1 and 2).

When the sequencing analysis was clustered into phylogenetic groups, there were representatives from the α-, *β*- and *γ*-subclass of *Proteobacteria*. The five groups in the water samples were 54.3% of the 'uncultured' group followed by 25.7% of AAPB (*α-Proteobacteria*), 11.4% of PSB (*γ-Proteobacteria*), 5.7% of PNSB (*α-Proteobacteria*) and 2.9% of PNSB (*β-Proteobacteria*) (Fig. 2A). The five clustered groups in the sediment samples were 38.9% of the 'uncultured' group followed by 27.8% of AAPB (*α-Proteobacteria*), 16.7% of PNSB (*α-Proteobacteria*) followed by 11.0% of PSB (*γ-Proteobacteria*) and 5.6% of PNSB (*β-Proteobacteria*) (Fig. 2B).

3.2. The relationship between DGGE profiles and Hg_T concentrations in sampling sites

From the data reported in Table 3, do they provide any information on the effects of the Hg_T concentrations in the water

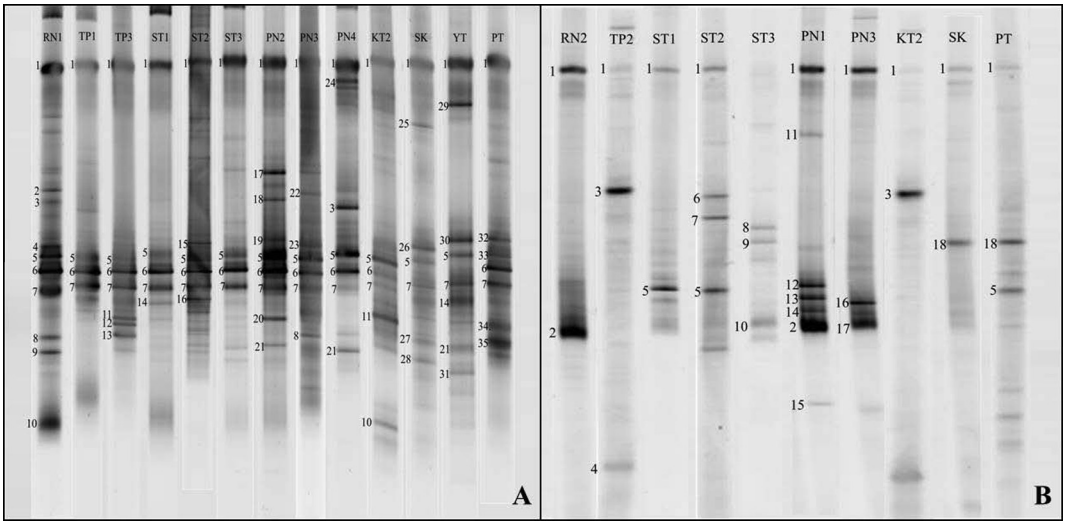

Figure 1 DGGE profiles of *pufM* amplified in bacteria from (A) water and (B) sediment samples collected from shrimp ponds located in the districts of Ranot (RN1–RN2), Tepa (TP1–TP3), Sating Phra (ST1–ST3), Pak Panang (PN2–PN4), Kantang (KT2), Sikao (SK), Yan Ta Khao (YT) and Mueang Pattani (PT). Marked numbers on the DGGE profiles represented bands that were excised and sequenced as shown in Tables 1 and 2.

Table 1 Similarity of representative *pufM* sequences in purple bacteria and AAPB from DGGE gels of water samples collecting from shrimp ponds in Southern Thailand.

Band	Sampling area	Closet identify relative		Similarity (%)
		Accession number	Bacterial species	
1	All area	CP000362	*Roseobacter denitrificans* OCh 114 (AAPB)	90
2	RN1	EU191249	Uncultured bacterium	95
3	RN1 and PN4	AM944092	*Rhodobium marinum* JA211 (PNSB)	92
4	RN1	EU191257	Uncultured bacterium	96
5	RN1, TP1, TP3, ST1, ST2, ST3, PN2, PN3, PN4, KT2, SK and YT	FN257160	*Halorhodospira halophila* strain H (PSB)	88
6	RN1, TP1, TP3, ST1, ST2, ST3, PN2, PN3, PN4, KT2 and PT	FR852763	*Marivita* sp. RCC 1921 (AAPB)	93
7	All area	JQ694099	*Roseibacterium elongatum* OCh 323 (AAPB)	93
8	RN1 and PN3	AB508087	Uncultured bacterium	88
9	RN1	CP000830	*Dinoroseobacter shibae* DFL 12 (AAPB)	89
10	RN1 and KT2	AB020784	*Rhodovulum sulfidophilum* (PNSB)	98
11	TP3 and KT2	FJ619033	Uncultured bacterium	90
12	TP3	HQ222675	Uncultured bacterium	90
13	TP3	AB486033	Uncultured bacterium	90
14	ST1 and YT	FN869946	Uncultured Rhodobacteraceae bacterium	90
15	ST2	EU009368	*Roseobacter* sp. BS90 (AAPB)	93
16	ST2	AY234384	*Rubrivivax gelatinosus* strain S1 (PNSB)	90
17	PN2	JQ694098	*Roseibacterium* sp. JLT1202r (AAPB)	93
18	PN2	JF523530	Uncultured Chromatiaceae bacterium	91
19	PN2	FJ498837	Uncultured bacterium	92
20	PN2	AM944100	*Ectothiorhodospira imhoffii*, type strain JA319T (PSB)	89
21	PN2, PN4 and YT	EU196353	*Loktanella* sp. NP29 (AAPB)	91
22	PN3	KF008154|	Uncultured bacterium	86
23	PN3	KC768183	Uncultured bacterium	86
24	PN4	DQ915720	*Roseovarius tolerans* strain NBRC16695 (AAPB)	90
25	SK	FJ498843	Uncultured bacterium	88
26	SK	HQ222683	Uncultured bacterium	90
27	SK	KC900142	Uncultured bacterium	91
28	SK	JN712795	Uncultured bacterium	91
29	YT	FJ812046	*Allochromatium renukae* strain DSM 18713 (PSB)	90
30	YT	FN257187	*Allochromatium* sp. MTCH3IM086 (PSB)	90
31	YT	FJ589120	Uncultured bacterium	90
32	PT	FR852765	Bacterium RCC 1908	87
33	PT	CP001029	*Methylobacterium populi* BJ001 (AAPB)	85
34	PT	KF008154	Uncultured bacterium	86
35	PT	FJ619028	Uncultured bacterium	90

and sediment samples on the PNSB diversity? The highest concentration of Hg_T detected in the water was from a sample collected from the KT2 site (0.030 μg/L) followed by sites TP2 and YT (0.004 μg/L) and TP3 (0.003 μg/L) while the Hg_T concentrations in other samples were less than 0.002 μg/L. In contrast, the Hg_T concentrations in the sediment samples were significantly higher than those in the water samples; and their Hg_T concentrations differed from different collecting sites. The Hg_T concentrations in the sediment samples ranged from 35.40 to 391.60 μg/kg dry sediment weight, with a median concentration of 166.01 ± 83.61 μg/kg dry sediment weight. The highest concentration of Hg_T was detected in the PT site while the lowest was from the PN3 site.

The DGGE profiles from the various sites of both sample types were compared by the Jaccard coefficient as shown by the UPGMA dendrograms in Fig. 3. The DGGE profiles of the water samples were clustered into seven groups (Fig. 3A). The DGGE profiles from sites; TP1, TP3, ST1–3 and KT2 were clustered in the first group; PN2 and PN4 were in the second group, and the remaining sites (PN3, RN1, SK, YT and PT) were each separated into an individual group. The DGGE profiles of the samples from the sediment were clustered into six groups (Fig. 3B). The DGGE profiles from sites; KT2 and TP2 were clustered in the first group and the ST1–2, PT and SK were in the second group, and the remaining sites (RN2, PN1, PN3 and ST3) were each separated into an individual group.

Comparison of the DGGE profiles in both sample types and their detected Hg_T concentrations in each sample showed that there was no relationship between the bacterial groups

Table 2 Similarity of representative *pufM* sequences in purple bacteria and AAPB from DGGE gels of sediment samples collecting from shrimp ponds in Southern Thailand.

Band	Sampling area	Closet identify relative		Similarity (%)
		Accession number	Bacterial species	
1	RN2, TP2, ST1, ST2, PN1, PN3, KT2, SK and PT	CP000362	*Roseobacter denitrificans* OCh 114 (AAPB)	90
2	RN2, PN1	JN712793	Uncultured bacterium	88
3	TP2 and KT2	FM208076	*Rhodovulum kholense*, type strain JA297T (PNSB)	90
4	TP2	AY853584	*Sphingomonas* sp. PB180 (AAPB)	90
5	ST1, ST2, and PT	FN257160	*Halorhodospira halophila* strain H (PSB)	88
6	ST2	DQ017882	*Methylobacterium rhodinum* strain ATCC 14821 (AAPB)	92
7	ST2	AB510456	Uncultured bacterium	89
8	ST3	JN712796	Uncultured bacterium	84
9	ST3	EU191583	Uncultured bacterium	87
10	ST3	AB510450	Uncultured bacterium	89
11	PN1	EU191602	Uncultured bacterium	92
12	PN1	FN257137	*Thiorhodococcus drewsii*, type strain DSM 15006T (PSB)	91
13	PN1	FJ498837	Uncultured bacterium	92
14	PN1	AY234384	*Rubrivivax gelatinosus* strain S1 (PNSB)	89
15	PN1	EU196353	*Loktanella* sp. NP29 (AAPB)	90
16	PN3	FR852763	*Marivita* sp. RCC 1921 (AAPB)	93
17	PN3	CP000613	*Rhodospirillum centenum* SW (PNSB)	89
18	SK and PT	AM944092	*Rhodobium marinum* JA211 (PNSB)	92

and Hg_T concentrations. For example, the sediment samples collected from sites; PT, ST1, ST2 and SK were clustered in the same group (Fig. 3B and Table 3), but the Hg_T concentrations (μg/kg dry sediment weight) were totally different as in PT (391.60), ST1 (367.68), ST2 (183.22) and SK (59.52). Moreover, the Hg_T concentration in RN1 (140.21 μg/kg dry sediment weight) was very close to that of RN2 (135.22 μg/kg dry sediment weight), but the RN1 site had no detectable *pufM* gene. This was similar for the water samples, as the DGGE profiles in KT2 site, with the highest concentrations of Hg_T (0.030 μg/L) was grouped with the TP1 and ST1–3 with the lowest concentration of Hg_T (< 0.002 μg/L). Therefore, the Hg_T concentrations were not in anyways related to the DGGE profiles in the sediment samples and also the water samples with a low Hg_T concentration.

4. Discussion

Although this research was initially focused on the PNSB the results showed that other groups of APB, PSB and AAPB, were also detected based on the amplification of the *pufM* genes (Tables 1 and 2 and Fig. 2). Our results are in agreement with the previous work that focused on the diversity of purple bacteria in a permanently frozen Antarctic Lake; however, in addition the purple bacteria, AAPB were also detected due to the use of the *pufM* gene probes (Karr et al., 2003). The AAPB that belong to the *α-Proteobacteria* (Fig. 2) were the group that showed varied species found in shrimp ponds in both sets of samples. This is not surprising as the *α-Proteobacteria* are the most abundant bacterial group in seawater (Sakami et al., 2008); and seawater is normally used for shrimp cultivation. In addition, aeration is always used in intensive shrimp ponds to improve water quality and increase shrimp yields; and all the 16 shrimp ponds studied were provided with mechanical continuous aeration, particularly at the end of culture. This is the reason why the AAPB such as *R. denitrificans* and *R. elongatum* were detected from all water samples; and *R. denitrificans* was detected from most sediment samples. The AAPB are aerobic and carry out anoxygenic photosynthesis by capturing energy from light using simple organic compounds as a carbon source for their growth (Yutin et al., 2007; Tang et al., 2009). In contrast, a greater diversity of the PNSB was found in the sediment samples than in the water samples with a higher detection rate of the *pufM* gene; this is because the sediment conditions in shrimp ponds are enriched with organic matter with only a limited amount oxygen and far from the sunlight, but the PNSB also grow in dark conditions using fermentation (Imhoff, 2001; Karr et al., 2003). Hence, PNSB could compete better with the PSB in the bottom of the ponds because light is limiting for photosynthesis. The high percentage of *pufM* amplified 'uncultured' bacteria in both sample types indicated that there could be many species of purple bacteria and AAPB in the shrimp pond environment that were uncultured species. In addition, it was possible that the extracted DNA template of these species were at low levels, thus resulting in a reduced-specificity for amplification of the *pufM* gene, so any incomplete sequence was classified as an 'uncultured' bacterium.

One interesting conclusion was that the anthropogenic organic matter that was used in shrimp feed was an important factor that influenced the bacterial community in shrimp ponds (Sakami et al., 2008). The feeding was provided based on the rate of diet feed, rate of shrimp growth and shrimp density that depended on the stocking density in a range of from 35 to 75 postlarvae/m^2 for the 16 shrimp ponds. The daily feeding rate for the shrimp cultivation in this study was in a range of 0.5–2 kg/10^5shrimps/day for an initial period and 5–6 kg/10^5shrimps/day just before harvesting. The sampling sites

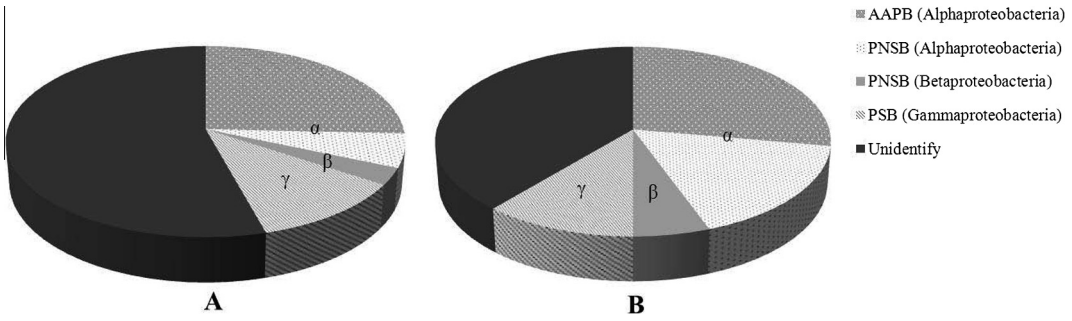

Figure 2 Phylum distribution of anoxygenic phototrophic bacteria sequences from (A) water and (B) sediment samples collecting from shrimp ponds in Southern Thailand.

Table 3 Group of DGGE profiles in water and sediment samples which were clustered by UPGMA dendrograms using Jaccard coefficient (Fig. 3) and comparing with Hg_T concentrations in water and sediment samples collected from shrimp ponds in Southern Thailand.

Site	Water		Sediment	
	Total mercury (Hg_T) (μg/L)	Group	Total mercury (Hg_T) (μg/kg dry weight)	Group
RN1	< 0.002 ± 0.001	4	140.21 ± 3.80	ND
RN2	< 0.002 ± 0.000	ND	135.22 ± 7.76	3
TP1	< 0.002 ± 0.000	1	169.42 ± 4.07	ND
TP2	0.004 ± 0.000	ND	204.50 ± 2.48	1
TP3	0.003 ± 0.001	1	243.56 ± 8.65	ND
ST1	< 0.002 ± 0.000	1	367.68 ± 15.87	2
ST2	< 0.002 ± 0.000	1	183.22 ± 1.87	2
ST3	< 0.002 ± 0.000	1	221.10 ± 0.75	6
PN1	< 0.002 ± 0.000	ND	52.32 ± 0.63	5
PN2	< 0.002 ± 0.000	2	63.94 ± 1.16	ND
PN3	< 0.002 ± 0.000	3	35.40 ± 2.55	4
PN4	< 0.002 ± 0.001	2	85.24 ± 5.51	ND
KT2	0.030 ± 0.004	1	165.92 ± 4.28	1
SK	< 0.002 ± 0.000	5	59.62 ± 4.45	2
YT	0.004 ± 0.001	6	87.26 ± 3.08	ND
PT	< 0.002 ± 0.001	7	391.60 ± 6.08	2

ND = not detected *pufM* gene.
Data represent mean ± standard deviation of three determinations.

that used similar shrimp feed for artificial diet (CP shrimp feed) although supplementary diet feeding was different produced a similar pattern of the APB community. For example, the DGGE profiles from the water samples collecting from the same district; TP1 and 3 (63 postlarvae/m^2) or different district; ST1–3 (75 postlarvae/m^2) were clustered in the same group (Fig. 3A), this result demonstrated that because these samples were from ponds with the same artificial diet, the type of shrimp feed in terms of quantity and frequency was similar in the period before harvest (5 kg/10^5 shrimps/day). Furthermore, the DGGE profiles of KT2 (Andaman coast) and TP2 (Gulf of Thailand coast) in the sediment samples (Fig. 3B) were clustered in the same group that had a different water source. However, shrimp feeding is not the sole factor that affected the diversity of purple bacteria because the DGGE profiles of the TP1 and TP3 from the same water source were clustered in the same group (Fig. 3A), but not from sediment samples as the *pufM* gene were not detected from both ponds (Fig. 3B). Hence, the stocking density of each pond might have an effect on diversity of APB during cultivation because of overfeeding so the uneaten shrimp feed was precipitated to the bottom of the shrimp ponds for stimulating APB growth. This is supported by the DGGE profiles from water and sediment samples of PN1 and 3 (stocking density, 35 postlarvae/m^2; feeding regime, 2 and 6 kg/10^5 shrimps/day for initial and before harvest phases) of detected *pufM* gene were different so they were in different clusters (Fig. 3A and B). These results pointed to the effects of shrimp feeding and stocking density on the APB found in the water and sediment samples, because the excess of the shrimp feed was precipitated to the bottom of the shrimp ponds to the more anaerobic conditions; particularly for PN1 and PN3. However, other factors that may affect the growth of APB include: pH, temperature, salinity, sanitizers such as chlorine, iodophors and the community of other bacteria in the shrimp ponds (Rao et al., 2000). Shrimp ponds that differed in these factors also showed a different diversity of APB as observed in the different sampling sites such as from RN1–2, SK, YT and PT (Fig. 3A) and RN2, PN3 and ST3 (Fig. 3B).

In this study we did not determine the physicochemical properties in the samples of water and sediment; however, the dominant species of detected APB could be used to support their roles on some of their physicochemical properties that were related to their characteristics. *R. denitrificans* was the most common species found in both sample types of all studied sites in the water column and almost all sites in the sediment; this organism is a photoheterotroph and also a denitrifying bacterium (Tang et al., 2009). Denitrification occurs in anaerobic conditions; but it is not strictly an anoxic process because nitrogen oxide reductases are expressed when some oxygen is present (Shapleigh, 2009). In addition, the research of Nishimura et al. (1996) found that *R. denitrificans* can grow in aerobic conditions because their photosynthesis genes are highly expressed in aerobic conditions. Hence, this is why this organism was found in both sample types. It is well recognized that the deposit of excess food in the sediment results in an anaerobic digestion process that produces NH_3 and H_2S that diffuse into the water column. Aeration in the water column helps the bacterial population to convert NH_3 to NO_3^- and H_2S to SO_4^{2-}. However, more PSB were found in the water column; this indicated that the sulfide (S^{2-}) produced in the sediment diffused into the water column. In shrimp ponds

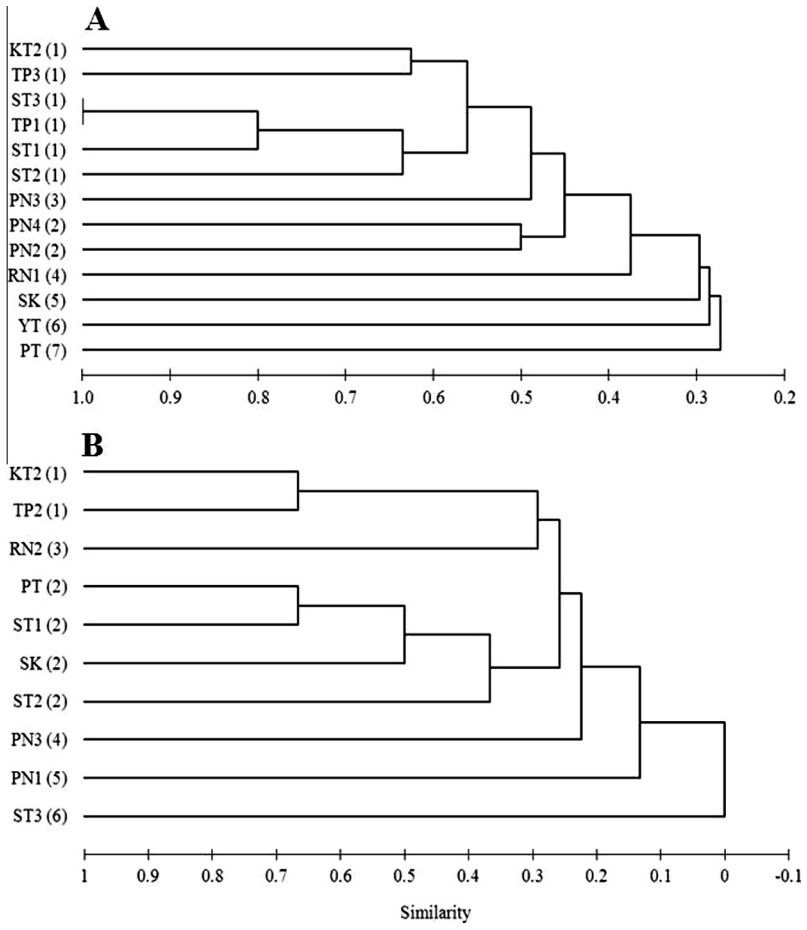

Figure 3 UPGMA dendrograms from cluster analysis using the Jaccard coefficient of DGGE profiles from (A) water and (B) sediment samples as shown in Fig. 1. Number in parentheses is a group of the clustered isolates.

the average concentration of SO_4^{2-} ranged from 1.6 to 4.3 g/kg, while the maximum concentration of S^{2-} was 1.5 mg/L (Mirzoyan et al., 2008). Therefore, under illuminated sulfidic conditions PSB grew by consuming S^{2-} as an electron donor for photoautotrophic growth although they are strictly anaerobes. This meant that bacterial communities in the water column including AAPB help to provide anoxic conditions for PSB to grow. In our study a high concentrations of H_2S in the shrimp ponds was assumed by the detection of the PSB species in the water samples (*E. imhoffii*, *H. halophila*, *A. renkae* and *Allochromatium* sp.) and sediment samples (*H. halophila* and *T. drewsii*) (Tables 1 and 2); and the sulfide should be completely consumed by them as evidenced by the healthy shrimp in the ponds as previously described. It is well recognized that *T. drewsii* found in the sediment and *R. sulfidophilum* (PNSB) found in the water samples were highly tolerant of the sulfide species, of up to 11 mM (Zaar et al., 2003) and 5–6 mM (Imhoff, 2005), respectively. Hence, this is a feasible biological method for controlling H_2S levels in the shrimp ponds via both groups of purple bacteria.

The toxicity of Hg on PNSB has been studied but only by an *in vitro* test i.e. Hg^{2+} at different concentrations (< 100 μM) inhibited the growth of *Rhodobacter sphaeroides* 2.4.1 by affecting the photosynthetic apparatus and the binding with C═O, C–N, C–S, and C–SH groups of the amino acids (Asztalos et al., 2012). In addition, Hg^{2+} at 0.03 mM gave a negative effect on *R. sphaeroides* R26.1 as the growth rate decreased while the lag-phase increased following the increase of the Hg^{2+} concentrations from 0.001 to 0.050 mM (Giotta et al., 2006). However, no work has been done on the effect of Hg on PNSB in shrimp ponds. The Hg_T concentration in the sediment was significantly higher than in the water column (Table 3). This was because most of the Hg in the water was attached to particles of suspended sediment, such as organic matter including metal oxides (Domagalski, 2001) and these precipitated onto the sediment. In addition, the Hg in the sediments was strongly bound to S^{2-} to form a highly insoluble, mercury sulfide (HgS) (Gabriel and Williamson, 2004).

The Hg_T concentration at each site was compared with the DGGE profiles (Tables 1 and 2) that were clustered by the UPGMA dendrograms (Fig. 3) as shown in Table 3. There was no direct relationship between both parameters (levels of Hg contamination and DGGE profiles) found, especially in the water samples, perhaps because the Hg_T concentrations in all water samples were very low. However, the Hg_T concentrations in the sediment samples were much higher than in the water samples, but there was still no correlation between the Hg_T concentration and the APB diversity. In contrast, use of soil microcosms spiked with inorganic Hg at 20 μg per gram

of soil had a significant effect on soil microbial communities (Harris-Hellal et al., 2009). Therefore, it is possible that APB, particularly PNSB have been acclimatized to live in long-term Hg contaminated shrimp ponds. This assumption is supported as more various species of PNSB were detected in the sediment samples with a lower number of *pufM* genes detected when compared with the water samples (Tables 1 and 2). Although it was not possible to find any correlation between the APB and Hg levels in the water samples as previously described; the results of the DGGE patterns of the *pufM* bands did change and opened up new avenues for discussion of the bacteria associated with shrimp cultivation. In addition, Hg contamination in shrimp ponds should be of concern because if there was no natural process to remediate and manage these areas, these will definitely increase especially in the sediment and might affect other beneficial microorganisms, and consequently perhaps the bioaccumulation of Hg in the shrimp via food chains. Hence, our future research work will focus on the various PNSB isolates to establish their Hg resistant mechanisms for the possibility of using them for remediation of Hg contaminated shrimp ponds and maintaining the quality of the shrimp cultivation water.

5. Conclusions

The amplification of APB based on the *pufM* gene found that the most common species found in the shrimp ponds were closely to *Roseobacter denitrificans* OCh 114 and *Halorhodospira halophila* H including some PNSB. These bacteria might play a major role in the nutrient cycles, particularly on the species of nitrogen and sulfur that are related to the water quality for shrimp growth. The Hg contamination in the shrimp ponds tested seemingly had no effect on the APB; in particular the PNSB.

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