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NGHIÊN CỨU ĐA DẠNG VI SINH VẬT Ở VỊNH NỔI TIẾNG VIỆT NAM BẰNG PHƯƠNG PHÁP SINH HỌC PHÂN TỬ HIỆN ĐẠI

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Tóm tắt: Trong công trình này chúng tôi nêu kết quả khảo sát số lượng và đa dạng vi sinh vật ở một số vịnh ven bờ như Hạ Long, Cát Bà, Dung Quất, Qui Nhơn, Nha Trang. Những khu vực gần bờ, gần cửa sông có số lượng vi sinh vật cao hơn, đa dạng hơn các mẫu lấy xa bờ. Số lượng vi khuẩn oxy hóa amonium và vi khuẩn sử dụng hydrocacbon là chỉ thị cho ô nhiễm chất hữu cơ và ô nhiễm dầu. Kết quả phân tích bằng phương pháp nuôi cấy và không qua nuôi cấy (DGGE) chứng tỏ vi sinh vật rất đa dạng, bao gồm vi khuẩn hiếu khí, nấm men, nấm mốc, xạ khuẩn, vi khuẩn lên men, vi khuẩn oxy hóa ammonium, vi khuẩn oxy hóa nitrit, vi khuẩn khử nitrat, vi khuẩn sử dụng dầu và vi khuẩn khử sunphat. Các vi khuẩn chiếm ưu thế thuộc về alpha và gamma Proteobacteria. Sử dụng phương pháp không nuôi cấy đã phát hiện một số vi khuẩn mới thuộc các chi *Acinetobacter*, *Rheinheimera, Alteromonas, Pseudoalteromonas, Rhodopirellula, Marinomonas, Microscilla, Brevibacterium* and *Cycloclasticus*. Vi sinh vật hữu ích trong các mẫu thu được đều xuất hiện với số lượng rất cao. Các số liệu thu được cũng cho thấy có thể phát huy tiềm năng của các vi sinh vật biển trong quá trình nuôi trồng thủy sản giúp hạn chế sự phát triển của các vi sinh vật gây bệnh và duy trì cân bằng sinh thái trong quá trình nuôi, đồng thời có thể ứng cứu khi có sự cố tràn dầu xảy ra bằng biện pháp phân hủy sinh học.

Từ khóa: *Đa dạng vi sinh vật, Proteobacteria, DGGE, Vi khuẩn hữu ích, Xử lý sinh học.*

STUDY ON MICROBIAL DIVERSITY AT VIETNAM FAMOUS BAY BY MOLECULAR MODERN METHODS

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Abstract: In this report, we show results on microbial diversity of some famous bays such as Ha Long, Cat Ba, Dung Quat, Qui Nhon and Nha Trang.

Some coastal areas and estuaries with high frequency of ship traffic had a higher amount of microorganisms than offshore areas. Numbers of ammonium oxidizing bacteria nda hydrocarbon utilizing bacteria are indicators for pollution of organic compounds and oil. Cultivation and non-cultivation methods (DGGE) clearly indicate that microorganisms were very diverse and include aerobic bacteria, yeast, fungi, Actinomyces, fermentative bacteria , ammonium oxidizing bacteria, nitrite oxidizing bacteria, nitrate reducing bacteria, hydrocarbon utilizing bacteria and sulfate reducing bacteria. The dominant specimen belong to alpha and gamma Proteobacteria. Using non-cultivation methods revealed some new bacteria such as *Acinetobacter*, *Rheinheimera, Alteromonas, Pseudoalteromonas, Rhodopirellula, Marinomonas, Microscilla, Brevibacterium* and *Cycloclasticus*. Useful microorganisms were encountered in all collected places with high quantities. Data also show that it is possible to use microorganisms for rearing food products in order to inhibit pathogenic microorganisms and maintain ecological balance. Additionally, microorganisms can be used in bioremediation of oil spills.

Key words: *Microbial diversity, Proteobacteria, DGGE, Useful bacteria, Bioremediation.*

I. INTRODUCTION

Microorganisms play an important role in carbon, nitrogen, phosphor and sulfur cycle. According to Zobell, predominant marine bacteria belong to *Pseudomonas*, *Vibrio*, *Spirillum*, *Achromobacter*, *Flavobacterium* and *Bacillus* (Atlas 1997). However, classical enrichment techniques often underestimate the bacterial diversity within with natural environment and only a small percentage of bacterial species have been isolated and cultured in the laboratory (Pace 1997). Recent publications concentrated on microbial community structure in different marine area in the world (North sea, Mediterranean sea, Pacific sea, Black sea, Arctic, Antarctic sea, Arabian , Spain, Japan sea) by modern techniques such as DGGE, TTGE (Urakawa 2000, Musat 2006 , Grote 2008, Kouridaki 2010, Marie 2010, Villaescusa 2010, Singh 2011).

In Vietnam, the activities of marine research was started from 1922. In recent years scientific researchers have collected many achievements of flora and fauna, contribute great deal for economic development of the country. However, the research on marine microorganisms is still very limited. Therefore, the investigation and conservation of microorganisms are necessary and urgent in order to evaluate microorganism source and conserve unique genes. Thereby, we should study thoroughly on environment, especially indigenous microorganisms in order to apply reasonable these microbes and balance the ecology. In this paper we focused on the number and diversity of microbial community in some famous bays such as Ha Long (Quang Ninh), Cat Ba (Hai Phong), Dung Quat (Quang

Ngai), Qui Nhon (Binh Dinh), Nha Trang (Khanh Hoa) and their role in environmental protection.

II. MATERIAL AND METHODS

Sampling sites/areas

Marine water samples were taken at Vietnam famous bays Halong, Dungquat , Quinhon, Nhatrang and Catba island from 4-8 m depth using Batometer sampler. Sediment samples were taken from 8-16 m depth using Peterson sampler (M 1200-C15 Wildco, USA).

Bacteria enumeration and isolation

Bacteria were enumerated and isolated from water or sediment samples on selective media: aerobic medium API RP38 for aerobic bacteria; MRS medium for fermentative bacteria; Postgate B medium for sulfate-reducing bacteria (Hien *et al*., 2003); Basruda medium for ammonia-oxidizing bacteria, Giltai medium for nitrate-reducing bacteria; Mineral Gost medium for hydrocarbon-utilizing bacteria; Hansen medium for yeast; Czapek Dox medium for fungi; Desoxycholate citrate agar; TCBS agar, LB agar and Modified Gause medium for *Streptomyces*. All media were supplemented with 20% (v/v) seawater.

Cell morphology and taxonomy

Morphology of bacteria was observed under Optical Microscope Laboval 4 (Germany) and electronic Microscope JEM 1010 (Japan).

Bacteria and yeasts were classified using a Biomerieux kit (API 20E, API 20NE, API 50CHB, API 50CHL and API20 CAUX) and based on 16S rRNA gene sequence analyses.

Streptomyces were classified according to Nonomura (1974). Fungi were classified according to Raper (1968) and Gams (1980). Bacterial diversity was studied using denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene sequence analysis (Paul 2001).

III.RESULTS

1. Number and distribution of microorganisms in investigated bays

1.1. Halong Bay

30 samples were taken from Halong Bay . The aerobic bacteria number was quite high, up to 10^6 CFU/ml water. Nitrogenous compound-converting bacteria were observed in all samples with 10^1-10^5 CFU/ml (or CFU /g sediment) (Fig. 1&2). Sulfate-reducing bacteria were present in almost all samples. In places near the shoreline, the number of SRB was up to 10^6 CFU/ml (or CFU /g sediment). These results indicate that Halong Bay is polluted. Moreover, the water from this area is threatened by some pathogenic bacteria such as Coliform (0-3x10^3 CFU/ml), *Vibrio* (0-5.5x10^1 CFU/ml), *Salmonella* and *Shigella* (0-1.9x10^1 CFU/ml).

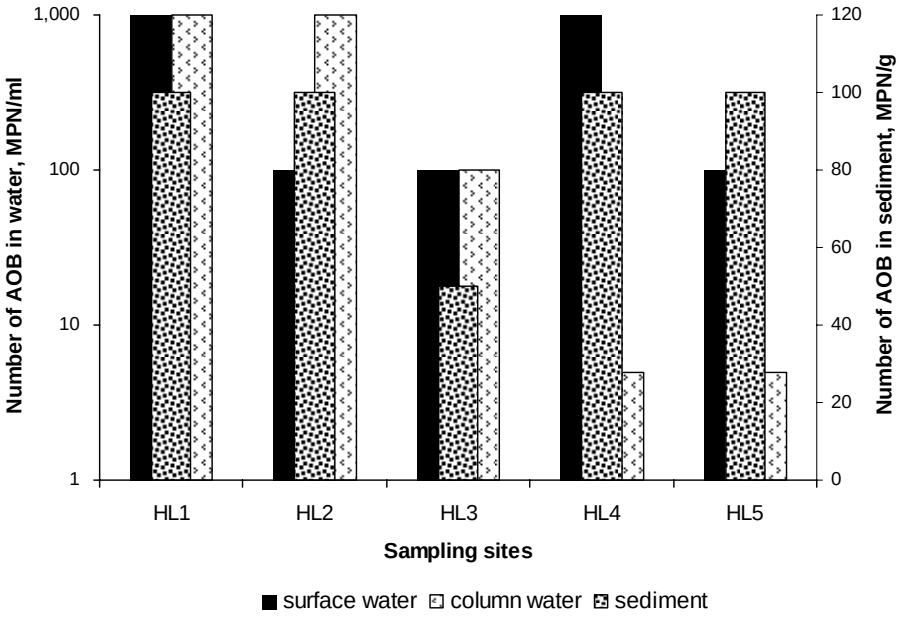

Figure 1: NH_4 oxidizing bacteria at different sampling sites in Ha Long Bay.

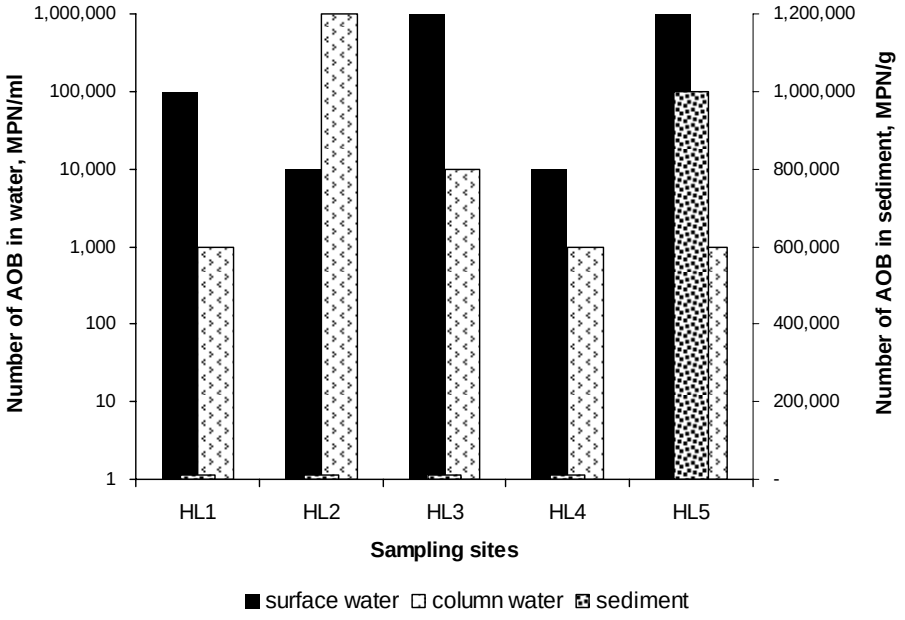

Figure 2: Aerobic bacteria at different sampling sites in Halong Bay.

1.2. Essential microorganisms in Catba Sea

Microorganisms were very diverse and included all essential groups such as aerobic bacteria (AB), yeast, fungi, *Streptomyces*, fermentative bacteria (FB), AOB, NOB, NRB, HUB, and SRB (Fig. 3).

The number of AB in the surface water was 10^5-10^6 CFU/ml; but was up to 10^7 CFU/g approximately in the sediment. FB were present in the surface water with 10^4-10^5 CFU/ml; but still 10-100 fold lower as compared to those in the sediment.

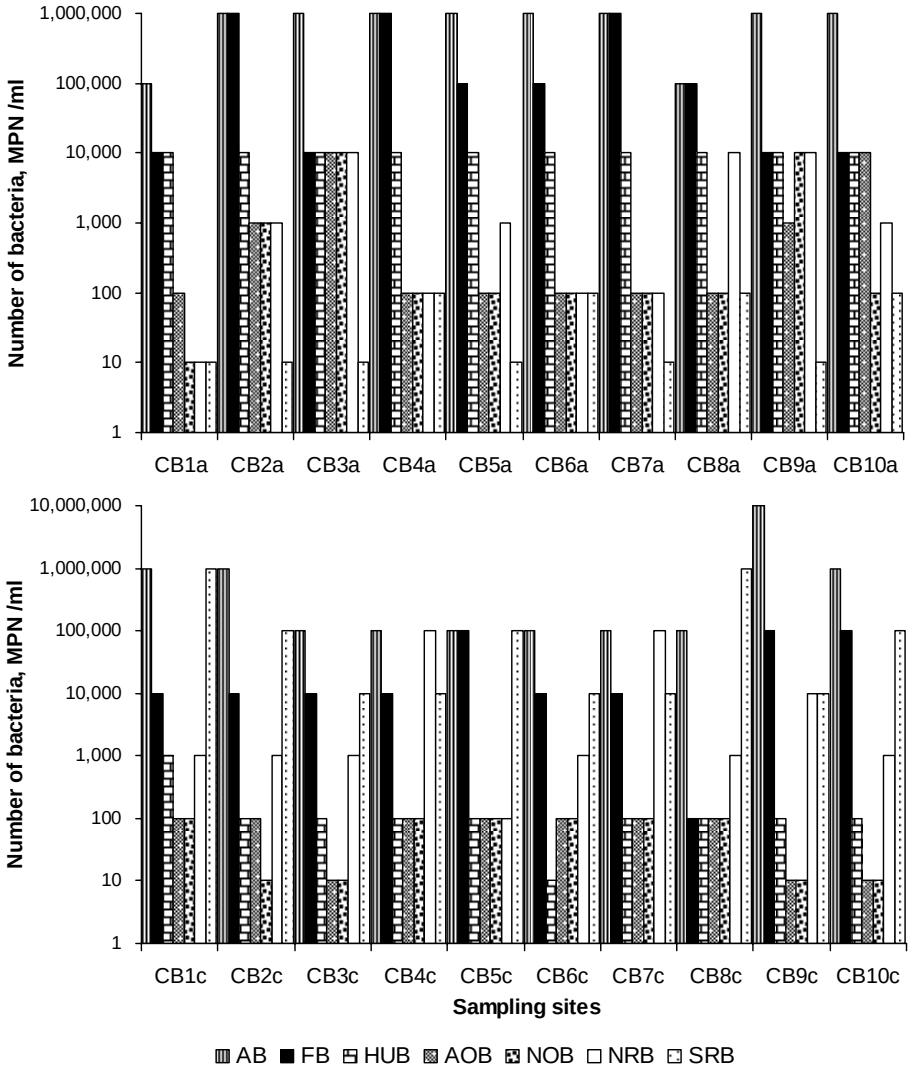

Figure 3: Number and composition of microorganisms at different sampling sites of Catba Island.

The quantities of AOB and NOB were 10^1-10^4 CFU/ml in the surface water and higher in the sediment, 10^2-10^6 CFU/g. The result suggested that the organic degradation process was intensive in this area. The number of NRB was $5x10^1$-10^4 CFU/ml in the surface water and much higher in the sediment, 10^2-10^6 CFU/g. HUB were present in the surface water with 10^4-5×10^4 CFU/ml, but 10 fold higher in the sediment. Such a high quantity of these bacteria suggests that hydrocarbon contamination of the Catba Sea has likely occurred. In these sand samples, the microorganism composition is diversified, and the aerobic and

hydrocarbon degrading microbial groups were predominant (Fig. 3). The numbers of these two groups were rather high, $5x10^5$ – $5x10^7$ CFU/g. Besides the above mentioned group of bacteria, SRB were also present with the quantity of 5×10^1-10^2 CFU/ml in the surface water, but very high in the sediment 10^4- 5×10^5 CFU/g. Fungi were observed in a quantity of 0-2×10^2 CFU/ml in the surface water but much higher in the sediment, at some places the number of fungi was even up to 8×10^4 CFU/g. No yeast was found in the sediment, but was present in few samples of surface water, with a quantity of 0-2×10^1 CFU/ml. In sample CB 4a, 6a-10a, *Streptomyces* was present in low quantity, 10^1 CFU/ml approximately (Fig. 3).

1.3. Essential microorganisms in Dung Quat

Dung Quat is one of the central bays, where the first oil refinery plant has been built. The number of aerobic bacteria was higher than that of anaerobic bacteria (10^4-10^7 CFU/ml compared to 10^1-10^4CFU/ml), especially in the water samples. The number of useful bacteria (hydrocarbon-utilizing bacteria and nitrate reducing bacteria) was rather high, even in the sediment samples (Fig. 4). The presence of pathogenic bacteria in many samples showed that water in this area is also polluted. The highest microbial numbers were aerobic bacteria (10^4-10^7CFU/ml), the lowest nitrification bacteria (0-10^2 CFU/ml). Numbers of harmful bacteria (SRB) were differing . In the same station, SRB in the surface water was less than that in the sediment. In contrast, aerobic and hydrocarbon-utilizing bacteria in the surface water were more than those in the sediment.

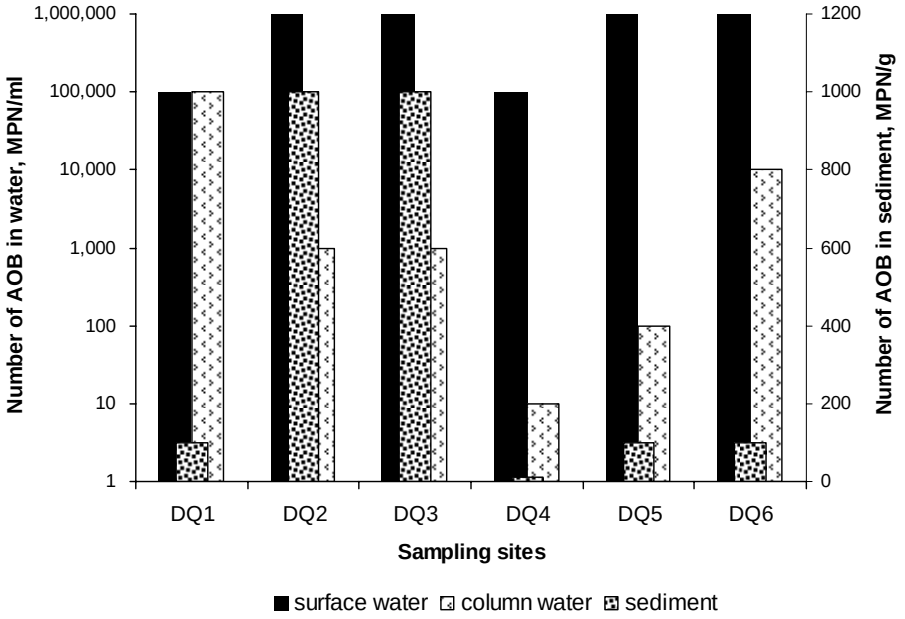

Figure 4: Hydrocarbon utilizing bacteria at different sampling sites in Dung Quat Bay.

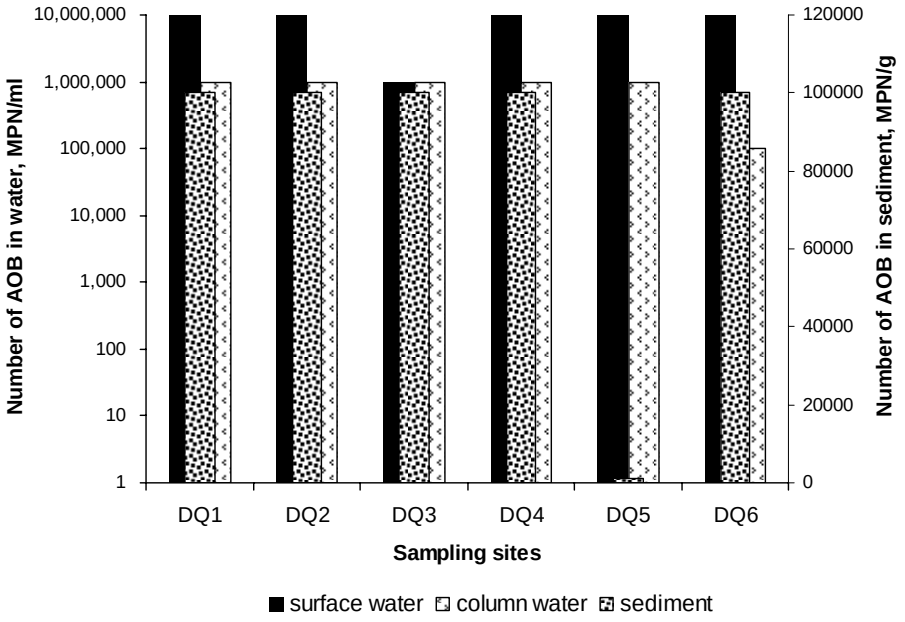

Figure 5: Aerobic bacteria (AB) at different sampling sites in Dung Quat Bay.

1.4. Essential microorganisms in Qui Nhon

In Qui Nhon Bay, the SRB number was higher than in the control sample, very far from the beach where there were not SRB. Water in Qui Nhon Bay is likely polluted from aquaculture.

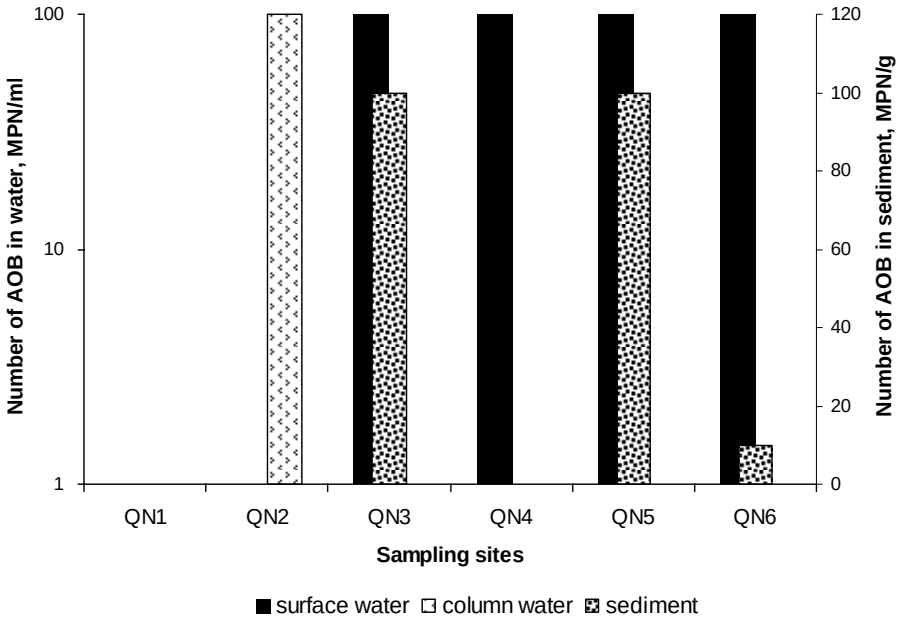

Figure 6: Pathogenic bacteria (PB) at different sampling sites in Qui Nhon Bay.

Analyzing 18 samples showed that samples QN-1, QN-2, QN-3, taken far from beach as control, had a lower pathogenic bacteria quantity than that in samples QN-4, QN-5, QN-6, taken from aquaculture area. Coliform bacteria existed in all samples with 10^1-10^2 CFU/ml (the highest is 1.4 x10^2 CFU/ml in sample QN-5c). Especially, *E.coli* and *Salmonella-Shigella* were present in almost all samples taken within Qui Nhon Bay, whereas they were rarely observed in samples near Culaoxanh, far from the beach. Therefore, Qui Nhon Bay is polluted not only by SRB, but also by pathogenic bacteria.

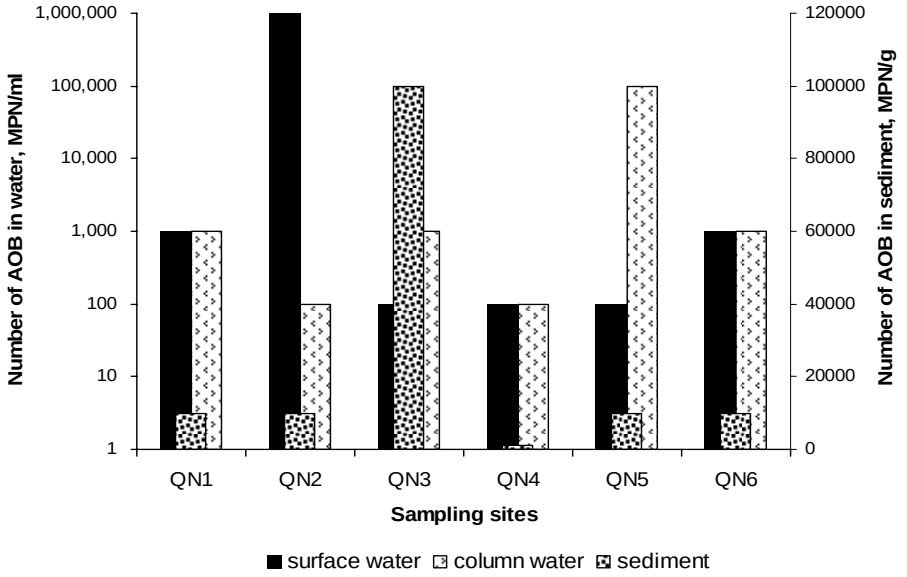

Figure 7: Sulfate-reducing bacteria (SRB) at different sampling sites in Qui Nhon Bay

1.5. Essential microorganisms in Nha Trang Bay

In Nha Trang samples, the numbers of AB, FB and HUB are very high up to 10^6 CFU/ml, while the number of nitrogen compound converting bacteria isvery low (Fig. 8 and 9). The SRB do not occur in some surface water samples. The results show that the environmental conditions are rather positive? and the bioremediation ability has a high potential. Among isolated bacterial strains, some of them were able to convert nitrogenous compounds, resistant to pathogenic bacteria and able to produce biosurfactants. These strains can be applied in aquaculture water treatment. Some predominant strains were classified as *Bacillus subtilis*, *B. pumilus, B. cereus, Nitrosomonas* sp., *Nitrobacter* sp., *Nitrococcus* sp., *Candida guiliermondiiand Rhodotorula glutinis.*

In conclusion, the distribution of microorganisms was very different according to the depth: in the sediment, the microorganisms number was higher from 10-1000 fold in comparison to the water at the sea surface. The beneficial microorganisms such as AB, FB, HUB, AOB, NOB, and NRB were encountered in all collected places with high quantities.

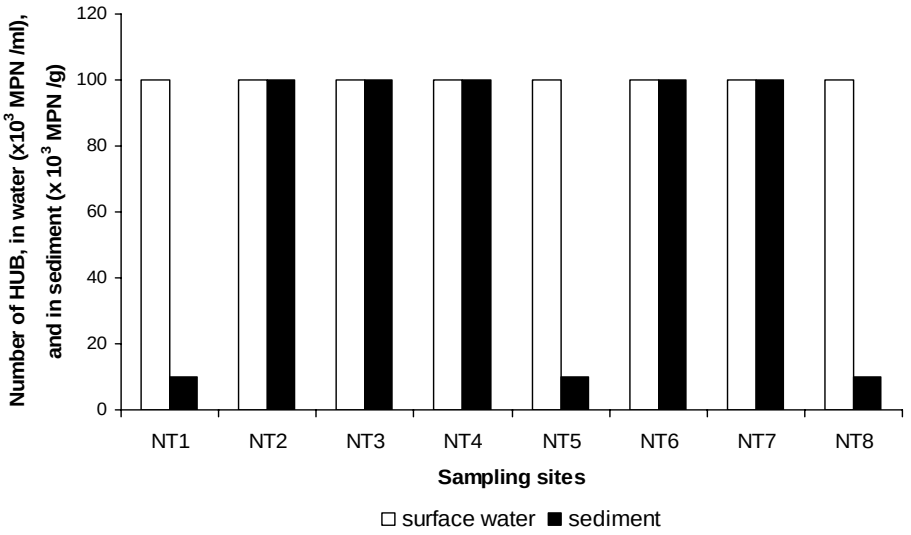

Figure 8: Hydrocarbon-utilizing bacteria (HUB) at different sampling sites in Nha Trang Bay

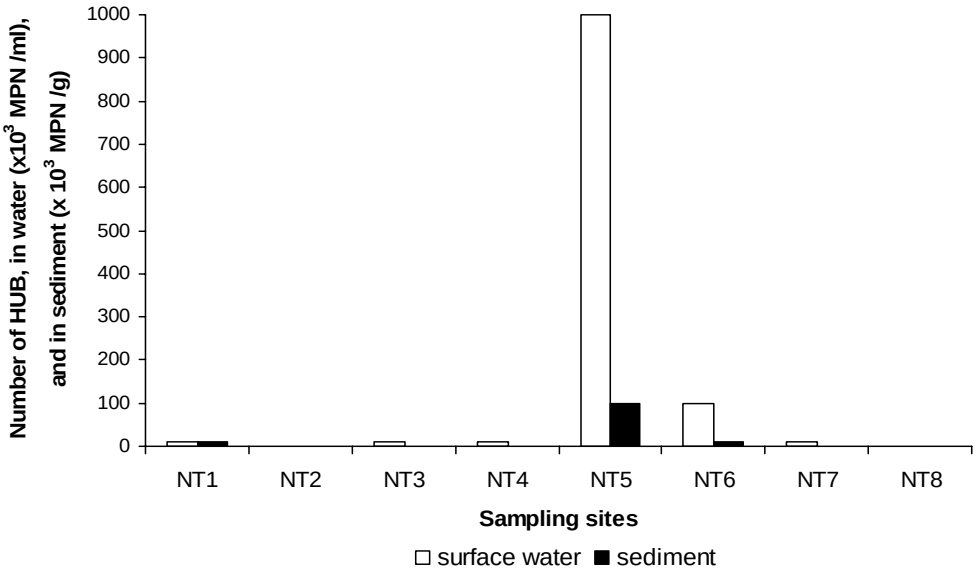

Figure 9: Nitrate-reducing bacteria (NRB) at different sampling sites in Nha Trang Bay

2. Classification of cultivated microorganisms

API chemical standard kits were used to classify the dominant bacterial and yeast strains, and combined with sequence analysis of 16S rRNA gene for those strains which could not be classified using the API kits. We found that Gram negative bacteria were more dominant compared to the Gram positive bacteria, and included genera *Acinetobacter, Pseudomonas*, *Sphingomonas*, *Ochrobactrum*. $Gram^+$ bacteria were only *Bacillus* and *Janibacter*. Some representative

microorganisms in Vietnam bays are shown in Tab. 1, and Fig. 10&11. Comparing their 16S rRNA gene sequences to those in the data base gave 93-100% homology. Some predominant strains with potential application in aquaculture were classified as *Bacillus subtilis*, *B. pumilus*, *B. cereus*, *Nitrosomonas* sp., *Nitrobacter* sp., *Nitrococcus* sp., *Candida guiliermondii* and *Rhodotorula glutinis*.

Table 1 : Representative microorganisms in Vietnam Bays

Strains	Area	Identified name	Method	Homology (%)
HL 1b	Ha Long	*Candida palmioleophila*	26 S rRNA	100
HL 1a	Ha Long	*Hortaea wernecki*	26 S rRNA	98
HL 2a	Ha Long	*Rhodotorula glutinis*	API 20Caux	99.5
HL 4a	Ha Long	*Rhodosporium diobovatum*	26 S rRNA	99
HL 10a	Ha Long	*Candida atlantica*	26 S rRNA	99
CB 1c1	Cat Ba	*Ochrobactrum cytisis*	16S rRNA	100
CB 1c2	Cat Ba	*Bacillus megatherium*	16S rRNA	100
CB1C3	Cat Ba	*Acinetobacter calcoaceticus*	16S rRNA	99.9
CB 2a	Cat Ba	*Janibacter marinus*	16S rRNA	100
CB 4a	Cat Ba	*Streptomyces globisporus*	16S rRNA	99
CB 5a	Cat Ba	*Candida parapsilolis*	26S rRNA-D1/D2	99
CB 7a	Cat Ba	*Rhodosporidium spearocarpum*	26S rRNA-D1/D2	99,7
CB 8a	Cat Ba	*Candida famata*	API 20Caux	99.9
CB 13a	Cat Ba	*Rhodotorula minuta*	API 20Caux	99.3
CB 9a	Cat Ba	*Lactobaciluss celobiosus*	API 50 CHL	99
CB 9C3	Cat Ba	*Aeromonas hydrophila*	API 20 NE	90
CB 11	Cat Ba	*Bacillus circulans 1*	API 50 CHB	98
CB 11a	Cát Bà	*Lactobacillus curvatus*	API 50 CHL	99
CB 11C2	Cat Ba	*Enterobacter cloacae*	API 20 NE	95
CB 11C3	Cat Ba	*Paenibacillus marserans*	API 50 CHB	99
CB 12a	Cat Ba	*Acinetobacter venetianus*	16S rARN	99.8
DQ 3b	Dung Quat	*Flavobacterium brevi*	API 20NE	99
QN 5c1	Qui Nhon	*Xanthomonas maltophila*	API 20NE	99
QN5c3	Qui Nhon	*Sphingomonas maltivorum*	API 20NE	99
QN6a	Qui Nhon	*Pseudomonas sp.*	API 20NE	99
NT1	Nha Trang	*Bacillus subtilis*	API 50CHB	99
XT1	Nha Trang	*Bacillus sphericus*	API 50CHB	99
XT2	Nha Trang	*Bacillus amus*	API 50CHB	99
CR1	Nha Trang	*Bacillus pumilus*	API 50CHB	98
CR2	Nha Trang	*Bacillus cereus*	API 50CHB	98

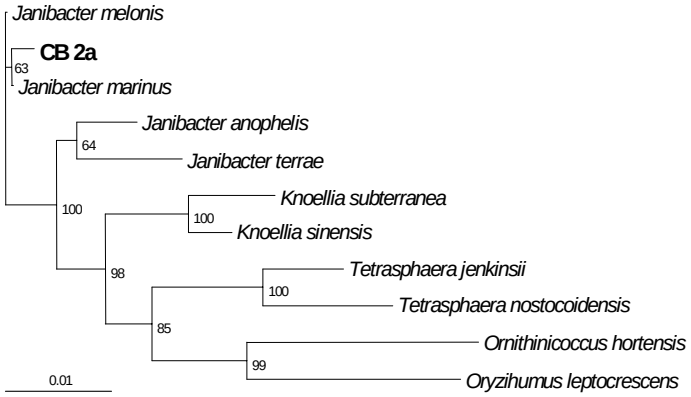

Figure 10: Phylogenetic tree of the bacteria-strain CB2a

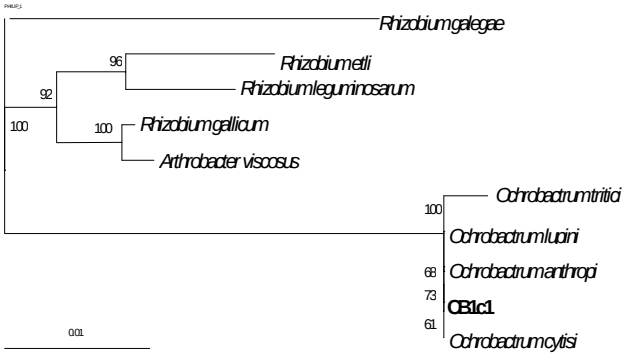

Figure 11: Phylogenetic tree of the bacteria-strain CB1c1.

3. Studying bacterial diversity by non-cultivation method (DGGE)

Denaturing gradient gel electrophoresis was used to separate the bacterial 16S rRNA genes amplified from the sea water, and then 16S rRNA genes were sequenced. The sequence comparison of 16S rRNA genes to the gene bank revealed that in sample CB2B, the bacteria included some members of genera *Rheinheimera, Pseudomonas, Alteromonas, Marinomonas, Acinetobacter* and *Rhodopirellula*. But in sample CB6B, *Rheinheimera, Marinomonas* were not found, but *Pseudoalteromonas, Ochrobactrum, Microscilla, Brevibacterium* and *Cycloclasticus* were present (Tab. 2). Thus, the diversity of bacteria in Vietnam sea is very specific. The dominant bacteria were Alpha and Gama Proteobacteria (Fig. 12). Comparing the non-cultivation to the cultivation method showed that most of the isolated bacteria were also determined by DGGE, suggesting a specific selectivity of the used media. However, we only selected the dominant isolated bacteria for further classification, so the results did not match the diversity of bacteria populations. Using non-cultivation methods helps to overcome the disadvantages of cultivation methods and revealed also new uncultivable bacteria, hence more bacteria were identified such as *Rheinheimera, Alteromonas, Pseudoalteromonas, Rhodopirellula, Marinomonas, Microscilla, Brevibacterium* and *Cycloclasticus*.

Table 2: Comparing the 16S rRNA sequences from marinebacteria with the data bank

Clone	Accession number	Species/Strain	Tentative genus	Homology (%)
CB2B_2	DQ298025	Strain K19414	*Rheinheimera*	97
CB2B_24	DQ298025	Strain K19414	*Rheinheimera*	97
CB2B_1	DQ227394	Strain E 4-1	*Pseudomonas*	99
CB2B_5	DQ227394	Strain E 4-1	*Pseudomonas*	99
CB2B_16	DQ227394	Strain E 4-1	*Pseudomonas*	99
CB2B_4	DQ452476	Strain NJ- 41	*Acinetobacter*	99
CB2B_15	DQ452476	Strain NJ- 41	*Acinetobacter*	99
CB2B_22	DQ452476	Strain NJ- 41	*Acinetobacter*	99
CB2B-132		*Rhodopirellula baltica*	-	
CB2B-20			*Alteromonas*	
CB2B-6			*Marinomonas*	
CB2B_18	AB101444	*Acinetobacter junii*	-	99
CB6B-30	AB101444	*Acinetobacter junii*	-	99
CB6B-31	AF529061	*Alteromonas marina*	-	99
CB6B-20	AB078014	Strain SHY1-1	*Alteromonas*	99
CB6B-27	AM409193	Strain 71(27zx)	*Pseudoalteromonas*	99
CB6B-32	DQ989292	Strain 1605	*Ochrobactrum*	99
CB6B-25	DQ989292	Strain 1605	*Ochrobactrum*	97
CB6B-28	AY386339	Strain HTCC208	-	95
CB6B-29	BX294149	*Rhodopirellula baltica*	-	95
CB6B-48			*Microscilla*	
CB6B-64			*Brevibacterium*	
CB6B-53			*Cycloclasticus*	

IV. DISCUSSION

Various studies have determined the presence of Gama, Delta proteobacteria, Flavobacteria, Plancomycetes in sediment from the North Sea, Arctic as well as in Antarctic Oceans. (Musat 2006, Villaescusa 2010). In sediments from Tokyo Bay, Japan, predominant bacterial groups are Delta, Gamma, Epsilon proteobacteria, Grampositive bacteria and Verrucomicroba (Urakawa 2000). When microbial populations in the deep ocean of the Nankai Through were investigated by DGGE method, Toffin et al. (2004) revealed that predominant bacteria were Firmicutes, Spirochaeta, Gamma and Epsilon proteobacteria. Castle and Kirchman (2004) reported that the composition of bacterial communities in Delaware estuary assessed by DGGE, contains alpha, beta, gamma proteobacteria

and cytophaga. The results of Singh et al. (2011) showed that dominant microbial populations in Arabian Sea are Gamma, Cyanobacteria and Bacteroidetes. According to Kochling et al. (2011) the microbial community of Cardiz Bay (Spain) were detected as Firmicutes, Delta, Gamma proteobacteria. This means the dominant marine bacteria in different Seas in the world are Delta, Gamma and Epsilon proteobacteria, while the Alpha and Gamma proteobacteria are predominant in Vietnam Seas only.

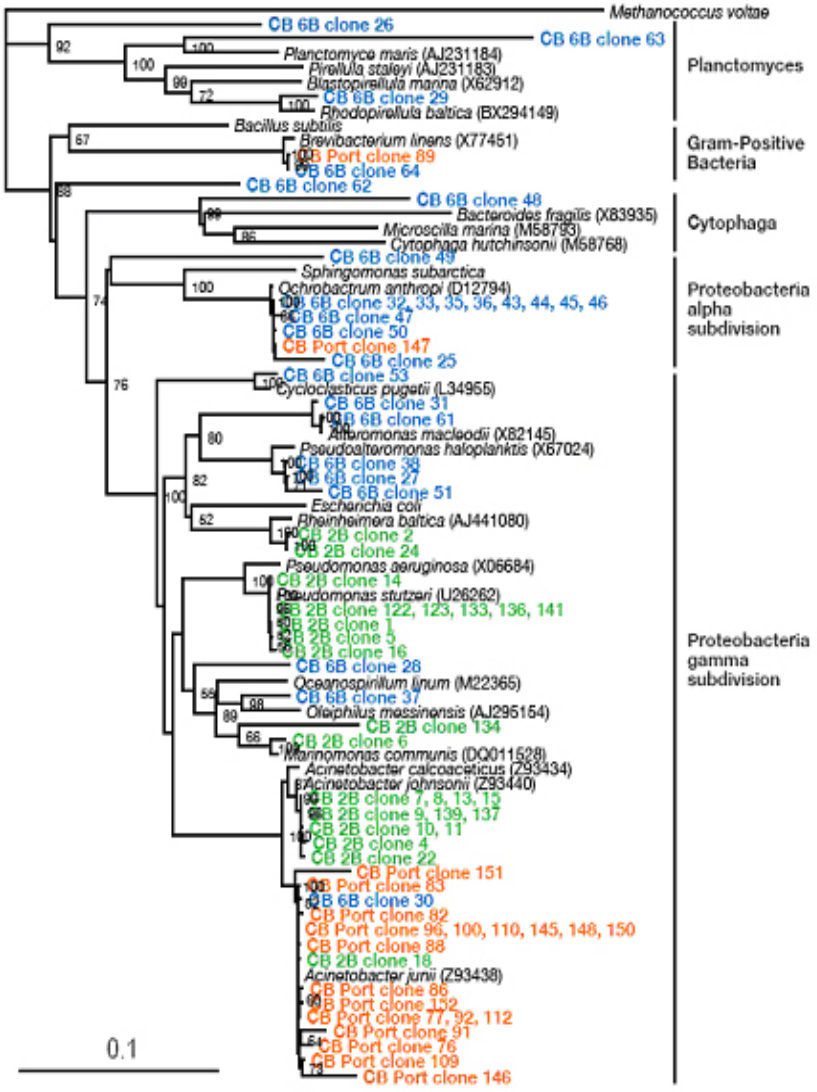

Figure 12: Phylogenetic tree exhibiting the bacterial diversity in marine water using the DGGE technique.

Results on microbial diversity in some Vietnam bays by cultivation and non- cultivation methods (DGGE) showed that microorganisms were very diverse and included aerobic bacteria, yeast, fungi, Actinomyces, fermentative bacteria , ammonium-oxidizing bacteria, nitrite-oxidizing bacteria, nitrate-reducing bacteria,

hydrocarbon-utilizing bacteria and sulfate-reducing bacteria. The heterotrophic bacteria are capable of utilizing hydrocarbon, lipid and protein as growth substrates in oxic and anoxic conditions.

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