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The impact of shrimp farming effluent on bacterial communities in mangrove waters, Ceará, Brazil

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Abstract

The effects of shrimp farm effluents on bacterial communities in mangroves have been infrequently reported. Classic and molecular biology methods were used to survey bacterial communities from four mangroves systems. Water temperature, salinity, pH, total heterotrophic bacteria and maximum probable numbers of *Vibrio* spp. were investigated. Genetic profiles of bacterial communities were also characterized by polymerase chain reaction (PCR) amplification of eubacterial and *Vibrio* 16S rDNA using denaturing gradient gel electrophoresis (DGGE). Highest heterotrophic counts were registered in the mangrove not directly polluted by shrimp farming. The Enterobacteriaceae and *Chryseomonas luteola* dominated the heterotrophic isolates. *Vibrio* spp. pathogenic to humans and shrimps were identified. Eubacterial genetic profiles suggest a shared community structure independent of mangrove system. *Vibrio* genetic profiles were mangrove specific. Neither microbial counts nor genetic profiling revealed a significant decrease in species richness associated with shrimp farm effluent. The complex nature of mangrove ecosystems and their microbial communities is discussed.

Keywords: Mangrove; Shrimp farm; Effluents; Bacterial community; DGGE

1. Introduction

Mangrove ecosystems are functionally complex, highly resilient and considered stable (Schaeffer-Novelli, 1999). They are thought to be among the most productive on the planet, fixing approximately around 2500 mg/year of carbon per square metre. Although they are rich in organic material, they are generally deficient in nutrients, in particular nitrogen and phosphorous (Vazquez et al., 2000). Mangroves are characteristic of tropical and sub-tropical regions where this type of ecosystem covers 18,107,700 ha of the planet; of which, 27.1% is in the Americas (Spalding et al., 1997). Brazil is home to the second largest area of mangrove in the World and the largest in the Americas. In Brazil, as elsewhere, over the last two decades there has been a substantial reduction in area covered by mangrove systems. This is because mangroves are being increasingly exploited near ports by industry, being developed as a result of urban spread and used to generate wealth through shrimp farming. Shrimp farming is seen as a key activity linked to change in habitat use, degradation and loss of mangroves. In some regions, large scale shrimp farming has already resulted in irreversible pollution and extreme salinisation (Phillips and Macintosh, 1997; Barg et al., 1997). Associated with shrimp farming are non-sustainable management practices involving the use of chemicals, antibiotics and exotic species which change and damage mangroves (Kautsky et al., 2000). In areas of intense shrimp farming, discharge waters from one farm mix with supply waters used by neighbouring farms resulting in pollution between farms and the spread

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of disease between shrimp populations (Smith, 1998). Research on the impact of shrimp farms on their surrounding environments so far indicates that effects are only detectable next to effluent discharge points, however, biological effects may be further reaching and are less well understood (Jones et al., 2001). Biao et al. (2004) mention that shrimp farm water quality and disease control are inter-dependent and to a certain extent are linked to microbial activity in these systems. There is however, an extreme lack of information, both qualitative and quantitative, about the effects of shrimp farm effluents on microbial communities. Torsvik et al. (1998) found that the genetic diversity of a mangrove, converted to shrimp production and then abandoned was 250 times lower than pristine mangroves. Torsvik's findings serve as a warning for those interested in shrimp production as well as those promoting the sustainable development of natural resources. If genetic diversity is desired but being significantly modified at what point is functional resilience lost? For mangroves like many other complex environments this is not yet well understood.

The northeast region of Brazil is responsible for around 97% of the national shrimp production. The increasing number and development of existing shrimp farms in this region has given rise to concerns about the effects of the discharge of effluent generated by the shrimp nurseries into the mangrove ecosystems. Despite the considerable amount of research evaluating the chemical and physical effects of these effluents on receiving water bodies (Burford et al., 2003) there have been few studies on the impact on the microbial component. Here we report on the bacterial communities of waters that had received shrimp farm effluent for "none", 5, 10 and 20 years. We surveyed the cultivable heterotrophic bacteria to assess broad scale microbial changes as well as cultivable isolates of the genus *Vibrio*. The genus *Vibrio* was chosen as a model group of microorganisms to assess the effects of shrimp farming effluent for a number of reasons: they are native to marine and estuarine environments used by shrimp farms (Thompson et al., 2004); some are opportunistic pathogens of shrimp (Saulnier et al., 2000; Sung et al., 2001), others are important human pathogens and associated with outbreaks of food poisoning from sea food (Gopal et al., 2005). In addition to surveys of cultivable bacteria, a culture independent genetic survey was made to assess if the mangrove waters receiving effluent waters showed signs of a dramatic decrease in genetic diversity as reported by Torsvik et al. (1998).

2. Materials and methods

2.1. Site description and water sampling

The Ceará coastline is situated between 2°46′–4°49′ latitude South (S) and 37°14′–41°19′ longitude West (W) stretching for approximately 537 km. The collection of samples took place in two consecutive years. In the first year, samples were collected from water from four mangrove swamps along the Ceará coastline: M1, *Rio Pacoti*; M2, *Rio Choro*; M3, *Rio Pirangi* and M4, *Rio Jaguaribe* (Fig. 1). M1 was used as a reference mangrove, without shrimp farm activity and effluent. Along the banks of the other three rivers, shrimp farming had been present for approximately 5, 10 and 20 years respectively. Samples were always collected at ebb tide and then immediately transported to the laboratory in sterilized dark glass flasks at 4 °C. At M1 two sampling points were chosen, P1 (3°49′9.5″S and 38°24′21.1″W) close to the river mouth and P2 up river (3°49′27.9″S and 38°25′8.3″W). At M2, M4 this pattern was repeated with M2P1 (4°6′07.1″S and 38°9′2.7″W) M4P1(4°25′29.5″S and 37°46′21.3″W) samples collected near the river mouth and M2P2 (4°6′17.3″S and 38°9′17.8″W) and M4P2 (4°27′59.6″S and 37°47′39.4″W) samples adjacent to shrimp farms. At M3 (4°24′4.9″ and 37°51′1″W) one sample site was selected which was adjacent to the shrimp farms.

2.2. Physical–chemical measurements

Water temperature and salinity were measured at the collection sites using a portable thermometer and refractometer (ATAGO S/MILL). The pH of the samples was determined in the laboratory using a potentiometer (MARCONI-PA 200P).

2.3. Total cultivable heterotrophic bacteria

The total heterotrophic bacteria (HB) count was determined on marine agar (MA) (APHA, 2000) medium using the spread-plate technique, in triplicate. After 48 h incubation the colonies were counted. Morphologically diverse colonies were isolated from each plate and sub-cultivated several times on marine agar medium. Isolated strains were stored in a fridge at ~4 °C in TSA medium (prepared with filtered seawater adjusted to 20% salinity with distilled water).

2.4. Vibrio counting and isolation

Vibrio spp. were counted and estimates of numbers made using the compendium of microbiological methods (Downes and Ito, 2001). Three colonies from each thiosulphate-citrate-bile-saccharose (TCBS) plate were selected and sub-cultured in MA medium for isolation of the strains.

2.5. Phenotypic identification of the isolated strains

The non-fermenting Gram negative bacterial strains were identified using the API 20NE kit (Biomerieux). Filtered and sterilized seawater, salinity adjusted to 20% with distilled water, was used instead of 0.85% saline solution. This change in method, based on a previous study by MacDonell et al. (1982) permitted a better characterization of

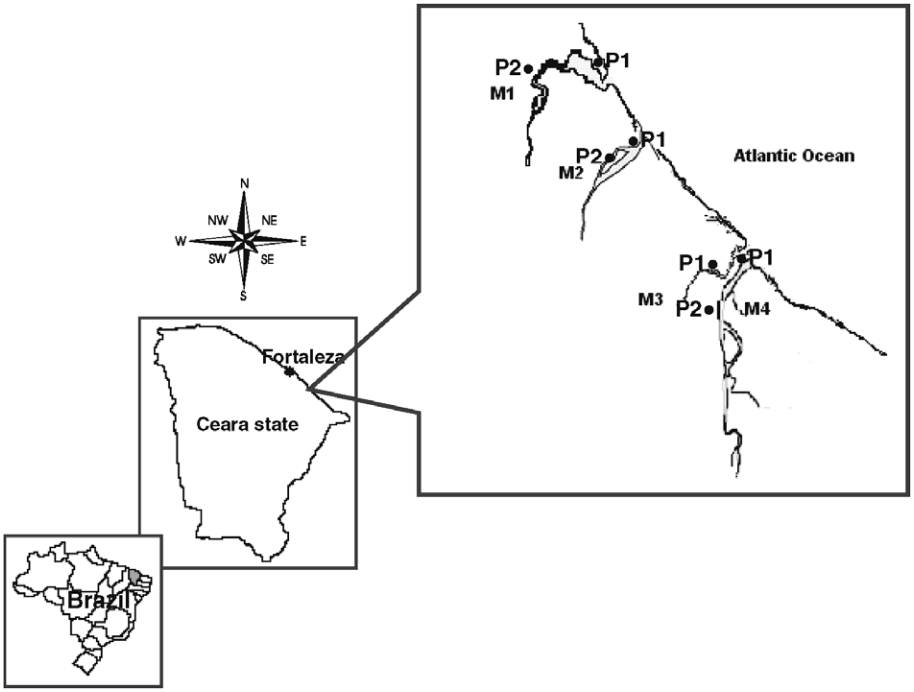

Fig. 1. Map of the study area on the east coast of Ceará state, Brazil.

the marine strains. All other strains were identified using phenotypic keys based on Bergey's Manual (1994) without modifications. The bacteria isolated from the TCBS plates (selective for vibrios), were further identified by biochemical tests described by Koneman et al. (1993) and Tison (1999).

2.6. Genomic DNA extraction

To investigate those microorganisms which did not grow on culture plates DNA was extracted from all life forms recovered on a filter through which 2 L of mangrove water had passed. The bacterial DNA fraction of total DNA was investigated by polymerase chain reaction (PCR) using reactions primed for vibrios and "all" bacteria – described later. Genomic DNA was extracted using the method of Petit et al. (1999). Two litres of mangrove water were filtered through a membrane (0.22 μm – Millipore) to collect the microrganisms. Immediately after the filtering, the membranes were placed in a denaturing solution (SDS 0.1%, Na_2EDTA 1 mM [pH 8.0], guanidine isothiocianate 0.5%) and frozen. The DNA was extracted from the filters using the protocol described by Sommerville et al. (1989) and modified by Rivera et al. (2003).

2.7. Primer design for PCR amplification of the Vibrio community

Novel *Vibrio* genus primers were designed to investigate this community from the "all life" DNA extracted from filtered mangrove water. Primers were designed against an alignment of published *Vibrio* and related 16S rRNA sequences and tested *in silico* with the Ribosomal Database Project (Cole et al., 2005; http://rdp.cme.msu.edu/) Probe Match program (version 2.0). The five primed end of forward primer was modified with a 30 base pair GC rich clamp (Myers et al., 1985). The newly designed primers were as follows: *vib-F*(727) 5′-AGG CGG CCC CCT GGA CAG A-3′ and *Vib-R*(1423) 5′-ARACTACCYRCTTCT-TTTGCAGC-3′. These novel primers share a 100% match percent with 346 of 363 sequences of the genus *Vibrio* (RDP: September 2005). PCR primers based on genes for ribosomal DNA (rDNA) are designed to specifically amplify selected groups of organisms from total community DNA. This molecular biology approach to ecology is considered universal as it can be applied to almost all life forms.

2.8. Amplification of "all" bacterial 16S rDNA from mangrove waters

All PCR amplifications were performed using a GeneAmp 9700 thermocycler (Applied Biosystems). The "all" bacteria community DNA, from the filters, was amplified using the following primers: F984 with GC-clamp (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and R1378 (5′-CGG TGT GTA CAA GGC CCG GGA ACG-3′) (Heuer et al., 1997) and *Vibrio* DNA was amplified using the primers described previously. Each PCR reaction mixture contained: 1x PCR buffer; 0.2 mM d-NTP's; 25 mM $MgCl_2$; 0.5% DMSO; 0.2 μM of each primer; 2.5 units Taq (Sttoffel Fragment, Applied Biosystem); 50 ng of the DNA template and 13.25 μL of distilled deionized water to a total reaction volume of

25 μL. Thermal cycling was as follows: one cycle: 7 min 94 °C; 30 cycles of: 1 min 94 °C, 1 min 56 °C, 1 min 72 °C, one cycle of: 10 min 72 °C, PCR products were checked using standard agarose gel electrophoresis and ethidium bromide staining (Sambrook et al., 1989).

2.9. Bacterial community genetic profiles

Denaturing gradient gel eletrophoresis (DGGE) is a finger print/barcode method which results in a gel with a series of banding patterns each representing an amplified community. Genetic analyses of the bacterial communities were conducted using the double denaturing gradient gel electrophoresis (d-DGGE) technique using acrylamide:bis-acrylamide, 37.5:1 and a 65% and 35% urea/formamide gradient combined with a 9–6% acrylamide gradient (Gelfi et al., 1997). Electrophoresis was carried out in a D-Code universal mutation detection system (Bio-Rad, Hercules, Calif.), using 1x TAE, at 60 °C, 200 V for 5.5 h. Gels were stained using the silver nitrate according to Sambrook et al. (1989). After staining the DGGE gels were digitalized and analysed. Presence/absence of bands on gels was used to estimate community richness. Similarity dendrograms were constructed using the BioDiversity Professional Beta statistic programme (McAleece et al., 1997) using the DICE similarity coefficient and an UPGMA grouping algorithm.

2.10. Statistical analyses

Microbial count data was transformed into $\log_{10}$ prior to comparison. Analysis of variance between the samples (ANOVA) was estimated and mean values compared using the Turkey test ($p < 0.05$) using Origin software.

3. Results and discussion

3.1. Physical and chemical parameters of the water

The mean and standard deviation for mangrove water temperature, salinity and pH are shown in Table 1. Salinity levels were found to be highly variable within sites and this was probably influenced by collection point location, tide and the volume of fresh/rainwater received at those points prior to collection. Not surprisingly salinity gradually decreased away from the coast. The only exception to this expected trend was at Point 2 of site M3 where the collection point was very close to an effluent discharge channel where increased salt levels from the effluent are likely to have affected the result. Shrimp farm effluents typically have salinity levels as high as 35‰ (Páez-Osuna et al., 1998) as such near to outlets higher salinity levels are expected and are indicative of shrimp farm pollution. The average water temperatures at M1 (no direct effluent), M2 (5 years of effluent), M3 (10 years of effluent) and M4 (20 years of effluent) were as expected and temperature increased slightly with distance from the ocean. The pH of the water varied little between the mangroves sites and between the points within each. The use of pH as indicator for shrimp farm pollution is complicated by a series of environmental factors. Boto and Bunt (1981) noted a strong correlation between the pH and the levels of oxygen dissolved in the water in a mangrove region in Australia; while Allan and Maguire (1993), Hopkins et al. (1993) and Shireman and Cichra (1994) all reported pH values which were significantly higher in shrimp nurseries than in effluent receiving waters next to farms. Abraham et al. (2004) found that temperature, pH and salinity of the supply and waste waters of shrimp farms were controlled by environmental factors such as solar radiation, the relative air temperature and rainfall. The first two factors encourage heating of the water during the day, provoking evaporation and an increase in the salinity values, rain reduces the temperature, pH and salinity of the water. In this study no clear correlation could be found between the physical–chemical characteristics investigated and the presence of shrimp farm pollution. This result indicates that the pollution caused by the shrimp farm effluent has been ameliorated by fluvial dilution to below detectable levels with the exception of higher salinity at Point 2, Mangrove 3.

3.2. Total heterotrophic bacteria counting and isolation

Heterotrophic bacterial (HB) counts are used as an indicator of water quality and qualitative data on the composition of a bacterial communities determined by

Table 1
Physical–chemical characterization of the mangrove sites investigated (Ceará, Brazil)

Sites	Temperature (°C)		Salinity (‰)		pH	
	P1	P2	P1	P2	P1	P2
1[a] Expedition						
M1*	29 ± 2.6	27.3 ± 2.1	13 ± 12.1	4.7 ± 8.0	7.83± 0.34	7.57 ± 0.21
M2*	28.6 ± 1.4	29.3 ± 1.2	0.8 ± 1.0	0.4 ± 0.5	7.70 ± 0.19	7.68 ± 0.20
M3*	29 ± 3.5	29 ± 3.6	10.4 ± 8.9	18.7 ± 1.5	7.32 ± 0.46	7.36 ± 0.40
M4*	31.3 ± 0.6	30.7 ± 1.2	27.3 ± 8.1	14 ± 9.6	7.93 ± 0.35	7.76 ± 0.43
2[a] Expedition						
M1**	31.2 ± 2.2		34.6 ± 0.5		7.82 ± 0.10	
M4**	26.4 ± 1.5		3 ± 3.7		7.30 ± 0.19	

* Mean of three samples.
** Mean of five samples ± one standard deviation.

Table 2
Number of heterotrophic bacteria at mangrove sites (Ceará, Brazil)

	Sites	M1	M2	M3	M4
1[a] Expedition					
Sample 1	P1	4.04 ± 0.03	4.33 ± 0.07	4.39 ± 0.07	6.92 ± 0.03
	P2	3.77 ± 0.03	5.24 ± 0.18	4.35 ± 0.04	4.89 ± 0.01
Sample 2	P1	5.83 ± 0.02	4.39 ± 0.04	5.67 ± 0.07	7.44 ± 0.05
	P2	4.38 ± 0.11	4.42 ± 0.06	6.82 ± 0.28	5.57 ± 0.00
Sample 3	P1	5.42 ± 0.12	5.33 ± 0.00	5.44 ± 0.08	4.41 ± 0.02
	P2	4.18 ± 0.10	6.49 ± 0.00	5.89 ± 0.08	6.89 ± 0.20
2[a] Expedition					
	P1	7.90 ± 0.07	nc	nc	4.06 ± 0.04
	P2	7.83 ± 0.06	nc	nc	4.15 ± 0.02
	P3	7.54 ± 0.00	nc	nc	5.85 ± 0.06
	P4	7.65 ± 0.04	nc	nc	4.44 ± 0.00
	P5	7.39 ± 0.00	nc	nc	4.34 ± 0.16

Values are $\log_{10}$ of CFU counts ± 1 standard deviation, nc – data not collected.

Table 3
Heterotrophic bacteria identified from mangrove waters (Ceará, Brazil)

Identification	M1	M2	M3	M4
Acinetobacter baumannii/calcoaceticus	2			1
Aeromonas sp.		1		
Agrobacterium radiobacter	2			
Brevundimonas vesicularis				1
Burkholderia cepacia			1	3
Chryseomonas luteola	4	1		2
Enterobacteriaceae	6	3		
Flavobacterium-Cytophaga sp.		1		
Ochrobactrum anthropi	1			
Photobacterium sp.				1
Pseudomonas paucimobilis	1			
Pseudomonas vesicularis				2
Vibrio/Aeromonas				1
Total number of isolates	16	6	1	11

HB counts are being established for a variety of specific environments (Edberg et al., 1997; Kalmbach et al., 1997; Norton and LeChevallier, 2000). The HB results reported here add to an expanding global database on bioindicators of pollution. In essence, the unifying characteristic of this group of bacteria is their general capacity for growth (formation of visible colonies) on many organic compounds (Reasoner, 1990) and that they occupy many ecological niches, in other words they are found everywhere (Begon et al., 1996). Heterotrophic bacterial counts for the mangroves investigated are shown in Table 2. The HB counts were found to be variable within and between sites. An expected trend of increased counts associated with sites near effluent rich in organic matter was not seen nor a temporal effect of higher counts from the sites which have been receiving effluent for 5, 10 and 20 years (Callisto et al., 2004). A second sampling expedition was undertaken and HB counts repeated for the mangrove without effluent and for the mangrove which has been receiving effluent for twenty years. This time, higher counts were seen from the site without shrimp farm pollution and not the long term effluent receiving site, a reversal on the first set of results. This reversal was unexpected, but not unusual when measuring microbial numbers in inherently dynamic environments such as estuaries and mangroves. Difficulties in sampling are confounded by difficulties in distinguishing between the estuarine populations and those which are carried from adjacent areas. The data we report demonstrates that in this study HB counts did not throw much light on understanding microbial dynamics in relation to shrimp farm pollution but rather gave a snapshot of numbers that vary significantly at a given sampling point and time.

3.3. Identification of cultivable heterotrophic strains

The majority of the isolated strains from M1 and M2 were identified as belonging to the Enterobacteriaceae family (Table 3). Enterobacteriaceae are indicators of sewage pollution and this result suggests that perhaps other anthropogenic sources of pollution are present and influencing the microbial communities at sites M1 and M2. The second largest number of isolates was identified as *Chryseomonas luteola*, which is in keeping with shrimp effluent pollution. Al-Harbi and Uddin (2004) report that *Chryseomonas* spp., *Vibrio* spp. and *Aeromonas hydrophila* are bacteria common in shrimp larvae cultivation systems. Al-Harbi and Uddin (2004) describe the genus *Vibrio* as being the dominant genus in such systems which supports the choice of this group as a bioindicator. According to Maeda et al. (2002), among the aerobic bacterial groups, species belonging to the *Pseudomonas*, *Vibrio*, *Acinetobacter*, *Alteromonas* and *Flavobacterium* genera are common in shrimp farmed waters. Although the waters tested in this investigation were not directly from the farms, the cultivable species identified, reflect what one might expect from a shrimp farm. The species identified, however, are not significantly different from what was seen at the site without direct effluent discharge M1. Species richness in Table 3 can be summarised as 6:4:1:7 for sites M1:M2:M3:M4 respectively with greatest richness seen in the 20 year site but more isolates identifiable at the shrimp farm effluent free site. Relatively few strains of the genus *Vibrio* were isolated on the non-vibrio specific HB medium, however, it was possible to isolate and identify a great diversity of vibrios using the selective TBCS medium (Table 4).

3.4. Estimates of the number and diversity of Vibrio spp.

The abundance of *Vibrio* spp. did not differ much between the samples from the reference mangrove (M1) and the other mangroves sites (Table 4). Inside the farm adjacent to M4, the number of *Vibrio* spp. in the inlet channel was similar, as expected, to those in the river at M4 from which it is derived. The *Vibrio* spp. counts from within the nursery and outlet channel were approximately 10 times higher than those found in the river water indicating a considerable enrichment of vibrios caused by shrimp farming activity. Despite the smaller overall counts of *Vibrio* spp. at M1 and M2 they were more diverse than sites

Table 4
Maximum probable number of *Vibrio* spp. in mangrove waters (Ceará, Brazil)

Location	Samples		Environment	
	Point	Water (Log MPN/mL)	Temperature (°C)	Salinity (‰)
M1	P1	5.04	30.0	35.0
	P2	2.88	30.0	34.0
M2	P1	3.97	28.0	37.0
	P2	5.04	29.0	37.0
M3	P1	5.00	25.0	nd
M4	P1	5.04	26.0	nd
	P2	3.62	26.0	nd
Inlet channel		5.18	25.0	nd
Nursery water		6.38	25.0	7.0
Outlet channel		6.66	25.0	1.0

nd – Not detectable.

M3 and M4 which receive larger volumes of effluent from shrimp farms (Table 5). This would suggest that specific species within the *Vibrio* genus are enriched by shrimp farming but that separating changes in microbial communities caused by shrimp farming pollution and other anthropogenic pollution is difficult.

One hundred strains isolated on the vibrio TCBS selective medium, were characterized phenotypically. The number and names of species isolated from the TCBS plates are shown in Table 5. The greatest number of different species isolated was found at point M2P1. The second greatest number of species was detected in the water from the shrimp nursery. It is interesting to speculate whether the differences in community structure between the different sites represent a steady state natural community at M2P1 and a shrimp farm community at the nursery (NW) collection point (Table 5). In addition to the isolates which are important pathogens for humans and aquatic animals, the genus *Vibrio* also includes species involved in nutrient cycling such as *V. harveyii* not to mention others capable of breaking down chitin, aromatic polycyclical hydrocarbons which are extremely toxic for the environment (Thompson et al., 2004). *V. harveyii* is known to be the principal pathogen of the shrimps *Litopenaeus vannamei* and *Penaeus monodon* (Austin et al., 2003), and has already been responsible for serious losses in shrimp production in the Philippines and other countries in Southeast Asia (Gräslund and Bengtsson, 2001). The species of interest to human health such as *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* were also found in this study and in another, see Sousa et al. (2004).

Table 5
List of *Vibrio* spp. identified from mangrove waters (Ceará, Brazil)

Species	M1*		M2		M3	M4		Shrimp farm		
	P1	P2	P1	P2	P1	P1	P2	Ic	N	Oc
Vibrio spp.	3	8	2	4			1	3	1	2
V. aestuarinus	3	2	1			2				
V. alginolyticus		1								
V. anguillarum										
V. campbelli				1	1					3
V. cholerae non-O1 and non-O139	2		3	2		1				1
V. costicola			2		1				1	
V. damselae		1	1	3					1	1
V. diazotrophicus										
V. fisheri					1					
V. fluviales	4	1	1	2		1				
V. gazogenes	1			3						
V. harveyii		1	1						1	
V. metschinikovii	1	1								
V. mimicus	1				2					
V. ordalii			4							
V. parahaemolyticus			8						1	
V. splendidus			1						1	
V. vulnificus				1					1	
V. cincinnatiensis								1	2	
Total number of isolates	15	15	24	16	5	4	1	4	9	7

Identification considered positive at 80%; Ic: inlet channel; N: nursery; Oc: outlet channel.
* Reference site.

Clearly *Vibrio* communities do respond to shrimp farming activity and enriched numbers are released into neighbouring mangrove waters. The capacity of mangroves to assimilate periodic discharges of effluent from shrimp farm has been discussed (Trott and Alongi, 2000) and depends on the water depth, size, hydrodynamics and vegetation. Using counts as an indicator and given that no significant difference in counts between sites M1–M4 was seen, it would appear that the waste assimilation capacity of these mangrove sites has not been exceeded. What continues to be difficult to predict is when assimilation capacity has been exceeded, risk to human health has been significantly increased and irrevocable damage has been done to the ecosystem Costanzo et al. (2004) and Torsvik et al. (1998). Given the methodological limits of culture based surveys, a combination of environmental indicators continue to be required. Choosing combinations of methods and then separating the signal from the noise in complex dynamic environments remains challenging. Standard water quality parameters reported here and elsewhere are an incomplete reflection of the ecological processes they represent and in estuarine mangroves, as we have shown, can be very variable. Over the last 10 years molecular biology techniques have been combined with culture techniques to address some of these challenges.

3.5. Genetic characterization of microbial communities

Culture independent/genetic methods for studying microbial communities provide a more complete picture of bacterial biodiversity by overcoming some of the limits imposed by culturing. Culturing based surveys limit our view of diversity primarily because of the selective pressures imposed by microbial growth in isolation and on or

in media unlike niches found in nature/non-laboratory environments. Culturing selects for the strains within a total community that are best adapted to growth under laboratory conditions (Amann et al., 1995; Brock, 1987). The dominant species on the surface of an agar plate may reflect a minority population in the natural environment or vice versa, added to which the vast majority of species from complex environments will not grow on a "universal" medium. Relationships between population size within a microbial community and ecosystem service/function undertaken by a specific species remain to be understood. What can be said is that cultivation dependent strategies on their own skew interpretations of biodiversity and only in very specific environments can provide a reasonable measure of diversity (Pinhassi et al., 1997; Rehnstam et al., 1993). Molecular biology techniques based on the characterization of nucleic acids provide a complementary view of microbial communities from natural environments. These techniques can avoid the selective sampling pressures inherent in cultivation and have proven to describe species richness significantly better than culture techniques. Ten percent of the 200,000 16S rDNA sequences available online (RDP, January 2006) come from culture studies, the remainder from total extractions of DNA from environments. Limitations to molecular biology approaches are also method dependent and those which rely on PCR amplification of community mixtures are influenced by DNA extraction and primer annealing during PCR. That said, DGGE has been used to provide community profiles in over 700+ publications since the year 2000 (Web of Science: http://portal.isiknowledge.com) and it is generally accepted that the profiles produced are a qualitative reflection of the most abundant DNA species extracted from a given environment (O'Donnell et al., 2005). Where 16S rDNA-DGGE is used to measure community profiles Yu and Morrison (2004) have shown that particular attention is required when choosing variable regions for amplification. Given an understanding of the limitations of culture and culture independent approaches studies using both, provides a more complete assessment of microbial communities as they occur in nature. The DNA extraction method used in this study and the novel *Vibrio* spp. PCR primers designed for this study were effective in that microbial community profiles could be generated by DGGE and analysed (Figs. 2 and 3, Table 6).

Looking at the "all" eubacterial 16S rDNA genetic profiles (Fig. 2a) the strongest bands are shared by all samples indicating that the most abundant 16S rDNA types (bacterial populations) were present in all waters samples tested. This suggests a shared underlying bacterial community structure in mangrove waters which is in contrast to Crump et al. (2004) ideas on a single bacterioplanktonic estuarine community and also in contrast with the HB count data presented earlier. Cluster analysis of the eubacterial genetic profiles (Fig. 3) reveals that at the Kingdom rank reproducibility of profiles from the sampling sites was good with intra-site similarity 95% similar or better. At this taxonomic resolution similarities between the genetic profiles do not appear to be related to shrimp farm effluent, salinity, pH or temperature. The vibrio genetic profiles, on the other hand (Fig. 2b), show commonality within each mangrove where the strongest bands may reflect underlying but different *Vibrio* communities. The strong banding pattern for M1, differs from M2, and both differ from M3. Patterns from M4, which feeds the inlet channel to a shrimp farm and the nursery share banding patterns, and differ from M1–M3 and the outlet channel which is unlike any of the other environments. The average of the number of bands in the DGGE gels obtained from the products of amplification of the DNA

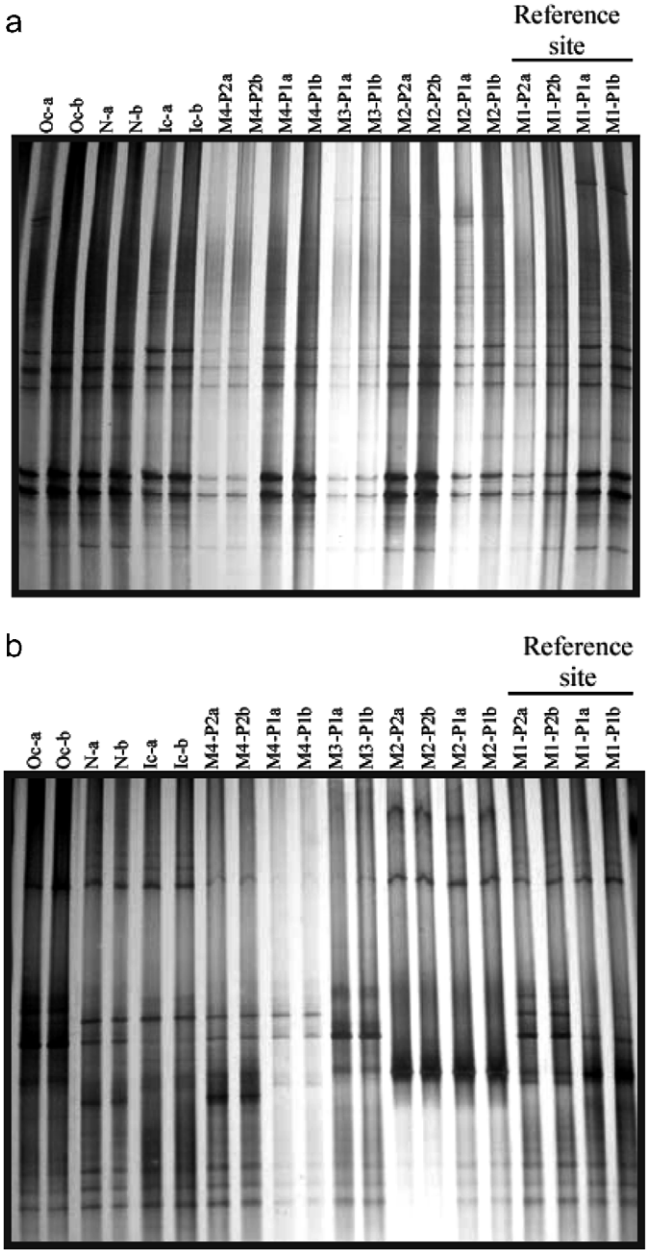

Fig. 2. Genetic profiles (a) Eubacterial 16S rDNA; (b) *Vibrio* 16S rDNA. Oc: outlet channel; N: nursery; Ic: inlet channel; M1 (reference site), M2; M3; M4 [sites that receive shrimp farm effluent, (Ceará, Brazil)]; letters a and b are replicas of the same sample.

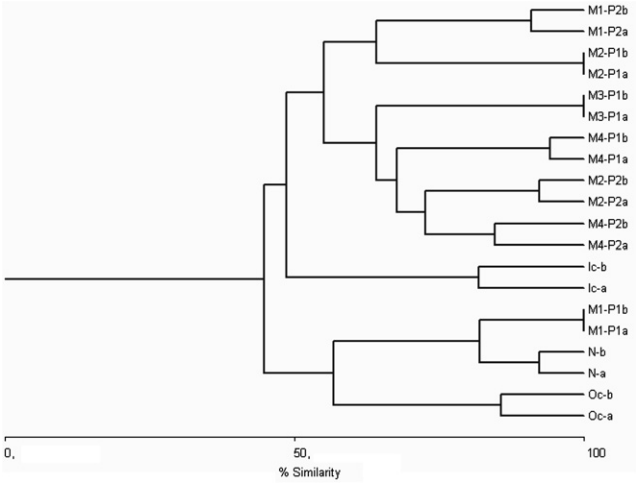

Fig. 3. Cluster analyses of Eubacterial 16S rDNA; Oc: outlet channel; N: nursery; Ic: inlet channel; M1 (reference site), M4; M3; M2 (sites that receive shrimp farm effluent sample (Ceará, Brazil); letters a and b are replicates of the same sample.

Table 6
Comparing numbers of DGGE bands with numbers of species identified from mangrove waters (Ceará, Brazil)

Samples	Eubacterial DGGE bands	Heterotrophic bacterial spp. isolated	Vibrio DGGE bands	*Vibrio* spp. isolated
Oc	20	nd	13	5
N	20	nd	13	8
Ic	7	nd	10	2
M4 P1	13	7	12	4
M4 P2	24	7	17	1
M3 P1	18	1	10	5
M2 P1	8	4	13	11
M2 P2	18	4	11	7
M1 P1	23	6	11	8
M1 P2	17	6	11	8

total of the water samples is shown in Table 6. Values varied from 7 to 24 for eubacterial profiles and 10 to 17 for the vibrios. It was possible to see a larger number of bands in the M4P2 and M3P1 samples. The average of the number of bands in the M4 sample, in the gel with universal initiators, was not significantly different from the control environment (M1) (ANOVA, $p > 0.05$). Owing to the variability of mangrove environments, correlations between bacterial profiles and shrimp farming effluent could not be reliably made and caution is required in order not to over interpret DGGE profiles without new corroboratory data. While the DGGE method provided mangrove specific community profiles, no relation between these and shrimp farm pollution could be established; likewise no apparent decrease in genetic diversity was seen in and between any of the sites investigated.

4. Final considerations

Based on the analyses of 10 sites in four mangroves, five sites from the *Rio Jaguaribe*, including an active shrimp farm we found that both the bacterial populations and composition of the microbial communities varied between mangroves and that *Vibrio* spp. counts increased in farmed waters. Genetic profiles at Kingdom rank indicate an underlying bacterial community structure which was ubiquitous to the four rivers and that studies targeted at this rank probably do not serve for bioindicator purposes. A genus specific study of *Vibrio* spp. returned diverse species of importance to shrimp farmers and of importance to human health. The genetic profiling of the vibrio group revealed mangrove specific communities but did not reflect changes associated with shrimp farms. The richness of isolated species, the number and difference in bands in genetic profiles (Table 6) do not suggest that any of the sites sampled have or are suffering from a large scale decrease in genetic diversity similar to that reported by Torsvik et al. (1998). The variety and combination of data types for the *Vibrio* group is positive indicating the potential of this group to provide species as bioindicators for the detection of shrimp farm effluent pollution.

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