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RAPD analysis of dominant denitrifying bacterial species in the estuarine environment of south west coast of India

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An investigation was made on the Random amplification of polymorphic DNA (RAPD) analysis of dominant denitrifying bacterial species in an estuarine environment (Rajakkamangalam estuary) of South west coast of India. From the collected water samples, the dominant denitrifying bacterial populations such as *Pseudomons aeruginosa* and *Bacillus cereus* were identified. These dominant denitrifying bacterial species were further characterized by using RAPD-PCR analysis. The results indicated that the amplification of polymorphic DNA of the above strains was varied much with respect to the variation in sampling stations and seasons. Accordingly, the similarity index and homology coefficient (%) of the isolates were also varied much.

[Key words: Monsoon, RAPD –PCR, *Pseudomonas aeruginosa*, *Bacillus cereus,* DNA]

Introduction

Polymerase chain reaction (PCR) can amplify the presence of organisms that cannot be cultured.[1] Ward[2] suggested that PCR amplification is a more specific test than DNA hybridization. The real time – PCR (RT-PCR) is developed based on traditional PCR.[3,4] In recent years, real time – PCR (RT-PCR) has been applied to study the abundance of microorganisms in soil, water and air.[5,6,7] Arbitrary / Random Primer Amplification of Polymorphic DNA (AP-PCR/RAPD-PCR) has been increasingly reported as a method for the genetic characterization of microorganisms. The RAPD technique, which was developed by Williams et al.[8] is being used to produce simple and reproducible DNA fingerprints in the presence of randomly designed short primers. Both genomic variations between bacterial species and genetic polymorphism between bacterial strains could be identified as the differences in the molecular size and number of DNA fragments obtained.

Molecular methods employed to study denitrifiers in the environment often start with PCR amplifications of the functional genes encoding the enzymes in the denitrifying pathway. The first primers targeting the functional genes encoding the enzymes involved in denitrification were published at the end of the 90's.[9] Scala and Kerkhof[10,11] reported that molecular approaches have been successfully used to detect and characterize bacteria and their genes that are important in several aspects of nitrogen cycle and denitrification. Application of molecular techniques to the study of denitrifying bacteria has been reported. Prime et al.[12] studied denitrifying bacterial communities in forest upland and wetland soils, and reported that in both sites, there existed a large amount of denitrifying bacteria and most of the *nir* genes detected were not found in the cultivated denitrifying bacteria. In addition, polymorphic RAPD fragments have also been used for the development of isolate and species specific probes for bacteria, fungi, protozoa and plants for distinguishing between closely related organisms.[13-18] Denitrification is also one of the most important ecological processes that encounter eutrophication.[19] Considering the information provided above, the RAPD-PCR was made on denitrifying bacterial population especially *Bacillus cereus* and *Pseudomonas aeruginosa* from estuarine environment.

Materials and Methods

Rajakkamangalam estuary is situated in Kanyakumari district at southern tip of Tamilnadu in the southwest coast of India. It is a bar built type. It is endowed with both southwest and northeast monsoons. Pannaivaikkal is one of the river systems that is found in this district. For the present study six stations from the estuary were selected for sampling (Fig.1). Station I is located in the sea, Station II located in the bar mouth region. Station III located near the Pannaiyoor Bridge and nearly 150 m away from station II; where as Station IV located 300 m away from station III and it is a freshwater zone. Station V located 200 m away from station III and it is a coconut retting effluent mixing zone. Station VI is a coconut husk retting zone and nearly 100 m away from station V.

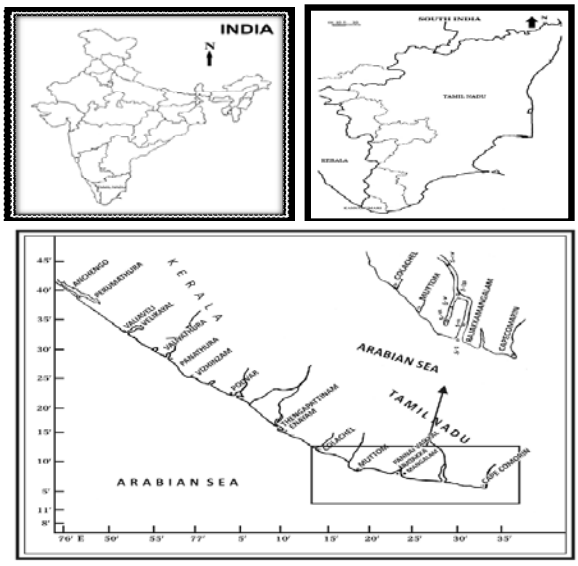

Fig. 1.Map showing study area and sampling stations (S-I to S-VI).

Surface water samples were collected from the representative months of non monsoon (March), southwest monsoon (July) and northeast monsoon (November) seasons during 2012 from the selected sampling stations of the experimental estuary during the early morning hours. The water samples were collected aseptically in sterile containers and were brought to the laboratory in an ice box. In the laboratory, one ml of water from each station was individually dissolved in 9 ml of sterile distilled water for further analysis.

In the laboratory, the water samples collected from the respective stations were serially diluted and then plated in Zobell marine agar as well as nutrient agar medium (Himedia, India) separately. The plates were incubated (37^{o}C) for 24h and the individual bacterial isolates were picked out based on the morphological characters. The bacterial strains isolated were then screened for their denitrification property. For this, a loop full of culture was taken and inoculated in nitrate broth present in the test tubes. After inoculation, the tubes were incubated for 48 to 96 h at 37ºC. Then 1 ml each of test reagents A (8.0 g sulphanilic acid in 1 L acetic acid) and B (5.0 g α- naphthalamine in 1 L acetic acid) were added. The formation of red colour indicated positive result. After screening the denitrifying bacterial strains, two dominant bacterial strains such as *Bacillus cereus* and *Pseudomonas aeruginosa* were identified by employing the scheme of Gunasekaran[20] and Holt et al.[21] The species level identification of *Pseudomonas* sp. was done on pyocyanin specific media. Here the growth and appearance of blue colour colony confirmed the *Pseudomonas aeruginosa.*[22] Similarly, the growth and appearance of pink, white, hallow colony on *Bacillus cereus* media confirmed the *Bacillus cereus*.[23] In the present study *P. aeruginosa* was recorded in the water samples of all the six stations, whereas *B.cereus* was recorded only in five stations except station I. These dominant *B. cereus* and *P. aeruginosa* strains were further characterized by using RAPD-PCR analysis.

B. cereus and P. aeruginosa cell pellets were ground separately in a glass homogenizer with 300 µl of CTAB (Cetyltrimethyl ammonium bromide) and DNA extraction buffer (1% W/V CTAB ; 1.4 m NaCl ; 10 mM EDTA, pH 8.0 ; 100 mM Tris-HCl ; pH 8.0 ; 0.2% V/V β-mercaptoethanol). The mixture was emulsified with equal volume of phenol: chloroform (1: 1). It was then centrifuged at 10000 rpm for 5 min. at room temperature. Aqueous phase was collected and mixed with equal volume of chloroform: isoamyl alcohol (24: 1). Mixture was again centrifuged at 10,000 rpm for 5 min. and the ethanol was air dried. Pellet was dissolved in 50 µl of TE buffer (Tris 10 mM, pH 8.0 and EDTA 1 mM, pH 8.0). The isolated DNA was quantified by Spectrophotometer (260 nm) and quality was tested by agarose gel electrophoresis.

Twenty µg of DNA was dissolved in 20 µl PCR reaction buffer containing 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.2 mM dNTPs, 21 pM of primer and 0.5 U of DNA polymerase. Ten primers (RAPD kit A1 to RAPD kit A10) obtained from IDT were used for

RAPD-PCR studies. PCR was performed according to the method of Williams et al. (1990) i.e., initial heat step (94^0C for 5 min), 40 cycles of denaturation (94^0C for 1 min), annealing (36^0C for 1 min) and extension (72^0C for 2 min) and a final extension step (72^0C for 7 min). Amplification was carried out using a programmable thermal cycler pre-150 (MJ Research USA). The products of PCR and DNA size markers [l µg DNA digested with test organisms and HindIII (Bangalore Genei)] were loaded onto a 1.6% Tris-borate EDTA agarose gel and ran for 4 h at 50V.[24] Gels were stained with ethidium bromide and photographed. Each lane of RAPD profiles was subjected to gel documentation system (Vilbert – Lourmal, France). Dendogram analysis and similarity index were carried out using Bioprofile ID software (Vilbert – Lourmal, France).

Primer code	Sequence
RAPD kit A1	5, CAGGCCCTTC 3,
RAPD kit A2	5, TGCCGAGCTG 3,
RAPD kit A3	5, AGTCAGCCAC 3,
RAPD kit A4	5, AATCGGGCTG 3,
RAPD kit A5	5, AGGGGTCTTG 3,
RAPD kit A6	5, GGTCCCTGAC 3,
RAPD kit A7	5, GAAACGGGTG 3,
RAPD kit A8	5, GTGACGTAGG 3,
RAPD kit A9	5, GGGTAACGCC 3,
RAPD kit A10	5, GTGATCGCAG 3,

Sequence of primers

Results

In the present study, RAPD analysis was performed for the dominant denitrifying bacterial species such as *P. aeruginosa* and *B. cereus* in the representative months of southwest monsoon, northeast monsoon and nonmonsoon seasons during 2012. The results indicated the existence of distinguished variation in the amplification of DNA base pairs of both *P. aeruginosa* and *B. cereus*. Further, sampling season's dependent variation in amplification of DNA base pair was also established well in the tested bacterial species.

For instance during the representative month (March, 2012) of non monsoon period, *P. aeruginosa* isolate 1 from S-I amplified five fragments of DNA with the base pair range of 244 to 3854. The isolates 2 to 6 from S-II to S-VI invariably amplified six fragments of DNA with the base pair range of 300 to 3850. Similarity index based on RAPD profile of these isolates indicated

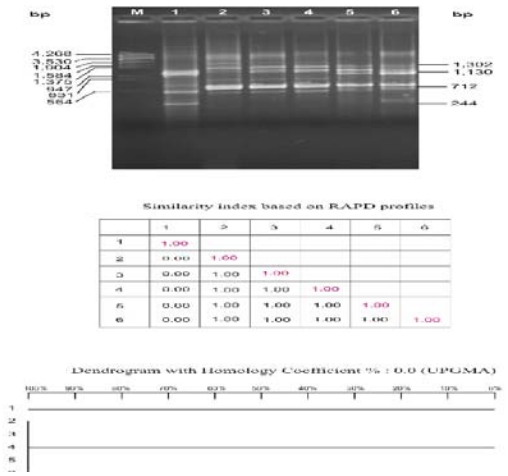

Fig. 2 - Random Amplified Polymorphic DNA of different isolates of *P. aeruginosa* isolated during nonmonsoon season in the selected stations generated by the primer RAPD kit (Lane 1 to 6 represents stations I –VI respectively).

the existence of the highest value of 1.0 between isolates 2 to 6. Isolate 1 indicated the nil index value with other tested isolates. Dendogram with homology co-efficient indicated the formation of single primary solidary clustered between isolates 2 to 6; whereas isolate 1 remained apart as a separate one (Fig. 2).

During this non monsoon period, *B. cereus* isolates 1 and 2 from S-II and S-III, amplified six fragments with the base pair range of 564 to 4360. Likewise, in isolates 3 and 5 from S-IV and S-VI, eight fragments with base pair range of 110 to 4260 were amplified. Isolate 4 from S-V amplified five fragments with the base pair range of 210 to 3530.

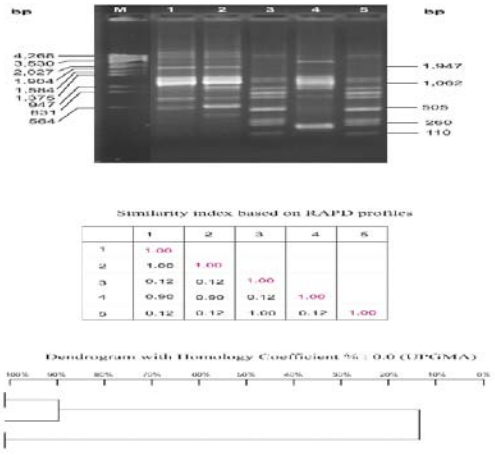

Fig. 3 - Random Amplified Polymorphic DNA of different isolates of *B. cereus* isolated during nonmonsoon season in the selected stations generated by the primer RAPD kit (Lane 1 to 5 represents stations II –VI respectively).

Similarity index based on RAPD profiles indicated the existence of the highest value of 1.0 for the relation between isolates 1 and 2 and also for isolates 3 and 5. The next highest value of 0.90 was noticed for the similarity between isolate 4 with isolates 1 and 2. The results on dendogram with homology coefficient (%) indicated that, isolates 1 and 2 formed single primary solidary cluster. Likewise isolates 3 and 5 also formed single primary solidary cluster. Isolates 4 formed secondary solidary cluster with isolates 1 and 2 (Fig.3).

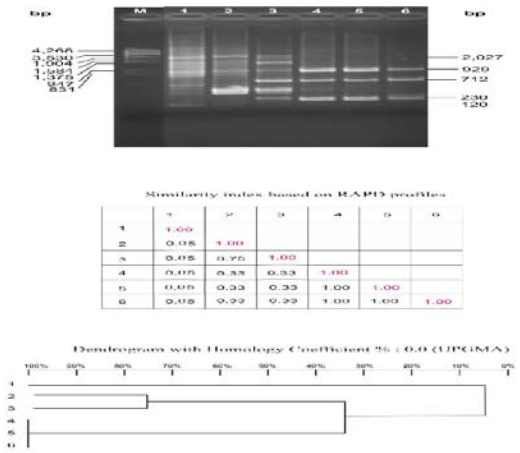

Fig. 4 - Random Amplified Polymorphic DNA of different isolates of *P. aeruginosa* isolated during southwest monsoon season in the selected stations generated by the primer RAPD kit (Lane 1 to 6 represents stations I –VI respectively).

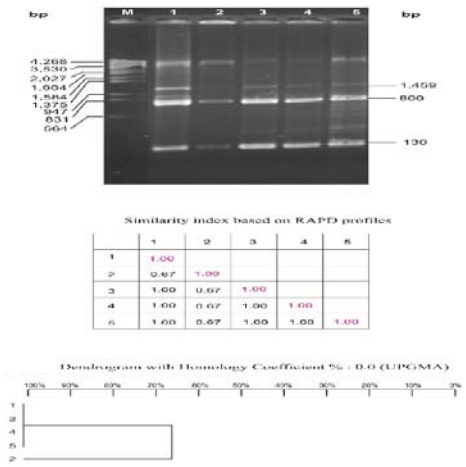

Fig. 5 - Random Amplified Polymorphic DNA of different isolates of *B. cereus* isolated during southwest monsoon season in the selected stations generated by the primer RAPD kit (Lane 1 to 5 represents stations II–VI respectively).

During the southwest monsoon period, (July,2012) the primer RAPD kit A10 amplified eight DNA fragments in the base pair range of 120 to 2027 in isolate 1 of *P. aeruginosa*. In isolates 2 and 3, five DNA fragments were expressed with the base pair range of 230 to 2027. In isolates 4, 5 and 6, only three base pairs with the range of 230 to 929 were registered. For this bacterial species, the results on similarity index indicated that, it was maximum (1.0) between isolates 4, 5 and 6. Next to this, the maximum similarity index value of 0.75 was noticed between isolates 2 and 3. The dendogram with homology co-efficient (%) indicated the formation of single primary solidary clustered between isolates 4, 5 and 6 and also between isolates 2 and 3 (Fig. 4).

During the same seasonal month (July 2012) in *B. cereus* four fragments were amplified in the base pair range of 130 to 4268 in isolates 1, 3, 4 and 5 respectively from stations S-II, S-IV, S-V and S-VI. In isolate 2 from station S-III, only three fragments were amplified with the base pair range of 130 to 4268. Results on similarity index indicated that the maximum value of 1.0 was obtained for isolates 1, 3, 4 and 5. The isolate 2 has the similarity index of 0.67 with isolates 1, 3, 4 and 5. Dendogram with homology co-efficient (%) indicated that isolates 1, 3, 4 and 5 formed single primary solidary cluster and isolate 2 formed secondary cluster with isolates 1, 3, 4 and 5 (Fig.5).

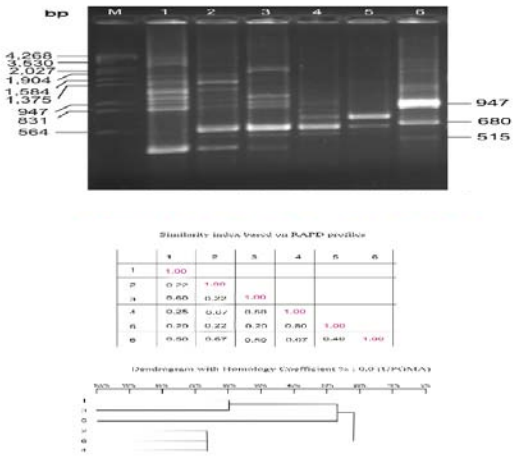

Fig. 6 - Random Amplified Polymorphic DNA of different isolates of *P. aeruginosa* isolated during northeast monsoon season in the selected stations generated by the primer RAPD kit(Lane 1 to 6 represents stations I –VI respectively).

During northeast monsoon season in November 2012, *P. aeruginosa* collected from S-I amplified eight fragments with base pair range of 220 to 3530. Isolate 2 from S-II amplified four fragments with the base pair range of 220 to 1620. Isolate 3 from S-III amplified four fragments with the base pair range of 564 to 2027. Likewise isolates 4 and 5 respectively from S-IV and S-V registered two fragments in the base pair range of 620 to 710. Isolate 6 from S-VI registered three fragments with the base pair range of 515 to 947. The results on similarity index based on RAPD profile indicated that, the highest value of 0.67 was noticed for the similarity between isolates 6, 2 and 4. The next highest similarity index value of 0.60 was noticed between isolates 1 and 3. Dendogram with homology coefficient indicated that isolates 1 and 3 and 4, 2 and 6 formed single solidary cluster. Isolate 5 formed secondary cluster with isolate 1 and 3 and also with isolate 4. Like wise isolate 6 formed secondary cluster with isolates 1 and 3 (Fig. 6).

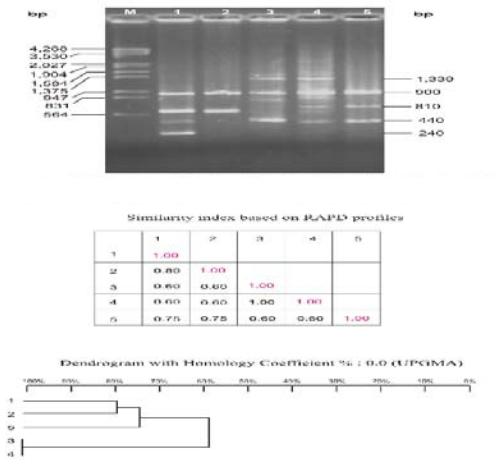

Fig. 7 - Random Amplified Polymorphic DNA of different isolates of *B. cereus* isolated during northeast monsoon season in the selected stations generated by the primer RAPD kit (Lane 1 to 5 represents stations II–VI respectively).

The DNA fragments amplified in *B. cereus* isolate 1 from S-II during the representative month of northeast monsoon season was four and the base pair range noticed was 240 to 900. More or less a similar variation was noticed for isolate 2 from S-III. Isolates 3 and 4 from S-IV and S-V amplified three DNA fragments with the base pair range of 440 to 1330. But isolate 5 from S-VI amplified three fragments with base pair range of 440 to 990. Similarity index based on RAPD profile showed the highest value of 1.0 and 0.80 between isolates 3 and 4 and 1 and 2, respectively. The next highest values of 0.75 and 0.60 were noticed for the relation between isolates 5, 1 and 2 and also with isolates 5, 3 and 4. The results on dendogram with homology co-efficient (%) indicated the occurrence of single primary solidary cluster between isolates 1 and 2 and also between isolates 3 and 4. Isolate 5 established secondary solidary cluster with isolates 1 and 2 and also with isolates 3 and 4. Likewise isolates 3 and 4 formed tertiary cluster with isolates 1, 2 and 5 (Fig. 7).

Discussion

Molecular genetic characterization of the experimental isolates, *B. cereus* and *P. aeruginosa* using PCR-RAPD technique was carried in order to assess the genetic heterogeneity and phylogenetic relationship among them. The potential of RAPD analysis for characterization of *B. cereus* and *P. aeruginosa* was also evolved. The results on RAPD analysis of dominant denitrifying bacterial species such as *B. cereus* and *P. aeruginosa* isolated from the water samples of the selected sampling stations of the experimental estuary indicated that the amplification of DNA base pair of both the tested species varied much and was found to be influenced by sampling stations and also by the sampling seasons. The tested isolate *P. aeruginosa* amplified 5 and 6 base pairs respectively in S-I and S-II to S-VI in March 2012 of non monsoon period. In the representative month of southwest monsoon (July 2012) *P. aeruginosa* isolated from S-I showed distinct pattern of DNA base pair amplification when compared with other isolates from S-II to S-VI. But the bacterial species isolated from S-I during representative month of northeast monsoon period (November 2012) showed similarity with the isolate collected from S-III. On the other hand the next dominant *B. cereus* was completely absent in S-I, which is being an exclusive marine environment. The same bacterial species isolated from S-II to S-VI showed a distinct variation in the amplification of DNA base pair and this is mainly due to the difference in the physicochemical properties of water and sediment present in that respective stations, which in turn influenced by both the monsoon seasons and

admixture of pollutants from the near by areas.

Pattanayak et al.[25] after comparing the results of earlier attempts to identify, characterize and discriminate between strains of *B. thuringenesis* by M13 finger printing, DNA hybridization using variable region of 16S rDNA, PCR finger printing and RAPD-PCR by Miteva et al.[26] Te Giffel et al.[27] Bourque et al.[28] and Pattanayak et al.[25,29] respectively, have concluded that fingerprinting based on M13, AFLP and RAPD were only able to detect high levels of diversity. They have also concluded that genetic relatedness and grouping of serovars based on RAPD analysis was similar to that of AFLP analysis. AFLP although very sensitive and powerful needed higher sophisticated technique and therefore recommended RAPD for characterization and classification in broad perspective to complement serotyping.

The banding pattern generated by the selected ten random primers in the dominant denitrifying bacterial species *B. cereus* and *P. aeruginosa* was further analyzed by Nel's homology co-efficient and dendogram, which indicated that these species also showed demarked variations in between sampling stations and also between sampling seasons. *P. aeruginosa* isolated from the sampling stations S-II to S-VI (isolates 2 to 6) during the representative month of nonmonsoon season (March, 2012) showed 100% similarity index with each other and shared single solidary cluster. This is the possible indication for the same species. On the other hand *P. aeruginosa* isolated from S-I (Isolate 1) showed 0% similarity index and formed a distinct group in the dendogram analysis. As reported earlier, S-I being the marine environment influenced the variation in DNA base pair and subsequently in similarity index and homology coefficient. This was the case for the isolate collected from the representative month (July, 2012) of southwest monsoon season. But the isolate collected during the representative month of northeast monsoon season (November, 2012) showed 60% similarity index with isolate 3 collected from S-III and shared single primary solidary cluster and there by indicated the same species. *P. aeruginosa* isolated from the representative month of southwest (July, 2012) and northeast monsoon (November, 2012) seasons from other stations (S-II to S-VI) showed season and station dependent similarity index and homology coefficient.

Similarly *B. cereus* isolated from S-II to S-VI (Isolates 1 to 5) except from S-I also showed variation in similarity index and homology coefficient. This variation in similarity index and homology coefficient of *B. cereus* (Isolates 1 to 5) was found to be influenced by variation in sampling stations and seasons. Isolates 1 and 2 of *B. cereus* collected from the representative month of non monsoon season (March, 2012) showed 100% similarity index and shared single primary solidary cluster in the dendogram with homology coefficient. This indicated that the isolates 1 and 2 may be the same species represented from S-II and S-III. More or less a similar trend was also noticed for the *B. cereus* isolates 1 and 2 collected from the representative month (November, 2012) of northeast monsoon season. But *B. cereus* isolate 2 from S-III showed only 0.67% similarity index with all other isolates (1, 3 to 5) collected from S-II, S-IV to S-VI during the representative month (July, 2012) of southwest monsoon season. The results clearly indicated the environmental dependent variation in genetic makeup of the characterized bacterial species. Smith et al.[30] quantitatively investigated the functional gene of denitrifying bacteria in estuarine sediment by RT-PCR, and found that the copy number of genes significantly decreased from estuary head to estuary mouth along a nitrate gradient. The application of Q-RT-PCR to environmental samples has been previously limited to quantification of bacterial gene expression in water.[31-34]

In an experiment involving RAPD analysis of *Acinetobacter* strains, by Koeleman et al.[35] showed that at least five different primers were needed out of the six, they initially used to produce optimal RAPD fingerprinting. However, in another experiment involving the typing of *Borrelia burgdorferi*, four primers were used and were said to be reproducible up to the 95% similarity level.[36] But in the present study ten different primers were used to produce RAPD finger printing.

Koeleman et al.[35] further inferred that although RAPD technique is a simple, rapid method of typing, its lack of reproducibility has been reported.

According to their study, it was due to the method's susceptibility to variation by primer and DNA concentration, DNA template quality (addressed above concerning contamination during the DNA isolation procedure), gel electrophoresis, and type of DNA polymerase. Standardization concentrations of purified genomic DNA, and standardized RAPD kits, helped to show RAPD as a reliable typing method. Holt et al.[37] differentiated *Leuconostic mesenteroid* strains according to the type of Dextran production. This study involved a modification of the RAPD-PCR method. The modification was to design RAPD primers, which would produce fragments within conserved sequences of certain genes to determine difference among identical species.

Smith et al.[30] indicated that the nitrate and nitrite reducing communities present in sediments from the Colne estuary are phylogenetically diverse and also divergent both from those in other environments and from cultured bacteria that are considered models for nitrate reduction. They have also developed and applied Q-(RT) - PCR to quantity specific nitrate/nitrite-reducing phylotypes along the Clone estuary's nitrate gradient and have demonstrated that gene copy and transcript numbers are, in general, greatest at the estuary head (Hythe), where the rates of denitrification / DNRA are highest.

Conclusion

Therefore in the present study, it is evident that both the sampling seasons and stations had a significant influence and may be the reason for the variations in amplification of the DNA base pair, related similarity index and homology coefficient of the dominant denitrifying bacterial species *B. cereus* and *P. aeruginosa* collected from the experimental estuary.

Acknowledgements

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