

Original Research Article

Temporal and spatial distribution of sulfate reducing bacteria in shrimp culture pond

sediment

Abstract

Sulfate reduction, a key process in aquatic sediments is carried out by a group of anaerobic microorganism called sulfate reducing bacteria (SRB). High numbers of sulphate reducers in shrimp aquaculture pond sediment deteriorates the soil and water quality, causing physiological stress, thereby reducing the immunity of cultured animal. An attempt was made to evaluate the temporal and spatial distribution of SRB horizontally at different locations viz., water pumping area (WPA), sluice gate (SG) and pond center (PC), and vertically from sediment water interface to 10 cm depth in Pacific white shrimp, *Penaeus vannamei* culture ponds. Physico-chemical characteristics of water and soil were correlated with the number of SRB. Distribution of SRB was significantly higher ($p \leq 0.05$) in the pond sediment nearer to SG (874 MPN/g), followed by PC (272 MPN/g) and WPA (99 MPN/g) and at sediment water interface (751 MPN/g) compared to 10 cm depth (114 MPN/g) of pond profile. Factors like dissolved oxygen and soil redox potential (oxidation to reduction scale), and organic carbon content in soil had a significant negative and positive correlation with SRB numbers. Phylogenetic relationship of SRB targeting SRB groups showed the presence of *Desulfonema*, *Desulfosarcina*, *Desulfatibaculum*, *Desulfobotulus*, *Desulfomicrobium*, *Desulfococcus*, *Desulfovibrio* and *Clostridium* genera in shrimp culture pond sediments.

Key words

Sulfate reducing bacteria, sediment-water interface, pond center, sluice gate, brackishwater and shrimp culture pond.

25 **Abbreviations**

26 SRB- Sulfate reducing bacteria

27 WPA-Water pumping area

28 SG- Sluice gate

29 PC- Pond centre

30 SWI- Sediment water interface

31 MPN- Most probable number

32 DO- Dissolved oxygen

33 OC-Organic carbon

34 TAN- Total ammoniocal nitrogen

35 OUT- Operational taxonomic units

36 DOC- Days of culture

37 **Introduction**

38 Pond ecosystem is a complex environment in which, microorganism present in water, sediment
39 and gut of the cultured animal interact each other which in turn has impact on growth, survival,
40 nutrient cycling and disease control (Cheng et al, 2010 & Zeng et al, 2010). Walker and Winton,
41 (2010) explained that poor soil and water quality along with bacterial and viral pathogens are the
42 main cause for mortalities in shrimp industry. High nutrients load derived from left over feed
43 and fecal materials that accumulate on pond bottom are a major concern, which leads to the
44 formation of toxic compounds like NH₃, NO₂ and H₂S. These toxic compounds deteriorate the
45 water and soil quality, causing physiological stress thereby reducing the immunity of cultured
46 animal (Wiyito et al, 2016).

In shrimp culture pond sediments, sulfate reduction is a major process which is carried out by a group of anaerobic microorganisms called sulfate reducing bacteria (SRB). This leads to the formation of hydrogen sulfide, a colorless gas with rotten egg smell and buildup of this toxic substance affects the shrimp health, thereby decreasing the production. Hsu and Chen (2007) documented that *P.vannamei* is more susceptible to *Vibrio* sp., and considerable reduction in immune parameters was found under sulfide stress. Generally in organically enriched sediments, the rate of sulfate reduction depends on factors like availability of organic compounds (Schubert et al, 2000), depth related gradient (Urakawa et al, 2000) and the quality and quantity of accumulated organic matter (Warner et al, 1934). Oxygen acts as a barrier for the movement of reduced toxic compounds into the water column (Boyd and Queiroz, 2014 & Wang et al, 2014). In aquatic sediments, as oxygen is consumed in 1-5 mm layer, sulfate reduction process becomes dominant in sediment water interface (SWI) and deeper layers (Meysman et al, 2010). All the factors mentioned above are not uniformly distributed and varies at different places in a pond and also from the neighboring ponds, the cause for variation in sulfate reduction and SRB population.

Several studies have focused on studying the diversity and composition of SRB in environments such as hyper saline lakes (Kjeldsen et al, 2007), freshwater (Mubmann et al, 2005), marine sediments (Purdy et al, 2001), estuarine sediments (Leloup et al, 2009) and aquaculture fish ponds (Kondo and Butani, 2007 & Kawahara et al, 2009). Few reports pertaining to the population of SRB in shrimp culture ponds are available with MPN (Most probable number) quantification (Suplee and Cotner 1996 & Abraham et al, 2004). In the previous studies, it was found that higher SRB in the range of $0.8-4.4 \times 10^9$ cells g^{-1} dry sediment was found in fish farm sediment and it was positively correlated with organic enrichment in the pond sediments

(Kawahara et al, 2008). Similarly, SRB in the range of 9,300–42,000 cm⁻³ was detected in the sediment of experimental ponds stocked with penaeid shrimp and fish (Rao et al, 2000). Other than these MPN quantification, studies on distribution of SRB in different places of the pond and their phylogenetic relationship in shrimp culture sediments are not available. The fact that only few studies are available with regard to SRB and its distribution in shrimp culture sediments, emphasizes the need for more studies on SRB and the role various pond environmental factors in influencing the process of sulfate reduction. Hence we made an attempt to study the phylogenetic relationship and distribution of SRB in shrimp culture ponds. In this study, temporal and spatial distribution of SRB horizontally at different places and vertically along the depth in brackishwater shrimp culture ponds. In addition to this, various pond environmental factors that influence the sulfate reducing bacteria was studied

2. Materials and Methods

2.1. Sample collection

Sediment samples were collected from three brackishwater shrimp culture farms located in Mamallapuram, Tamil Nadu, India. From each farm, four ponds were selected for this study. Pacific white shrimp *Penaeus vannamei* was the cultured species with a stocking density of 40 to 45 nos /m² in each pond and the culture period was 95 to 100 days. All the ponds followed zero water exchange culture with standard management practices. To study the horizontal and temporal distribution of SRB in the culture pond, sediment samples were collected from three locations viz., water pumping area (WPA), sluice gate (SG) and pond center (PC) at different days of culture (DOC) throughout the culture period (22, 37, 51, 65, 80 and the day of harvest). In addition, to study the temporal and vertical distribution of SRB, sediment samples were collected from the above mentioned locations at three time intervals (22 & 51 DOC and harvest

time, by inserting PVC core sampler from SWI and cores of 0-0.5 cm, 0.5-1.5 cm, 1.5-2.5 cm, 2.5-5.0 cm, 5.0-7.5 cm and 7.5-10 cm were separated. In order to have a uniform representative sample, soil samples of each core of same depth from all the sampling points in a pond were pooled together. All the samples were transported to laboratory in ice cold condition.

2.2. Sediment and water quality analysis

Measurement of pH, dissolved oxygen (DO) salinity and turbidity in pond water, and redox potential (E_h) in pond sediment was done onsite using multi-parameter water quality analyzer (Hach, USA) at all the sampling places. Water samples were analyzed for total sulfide-S, nitrite-N, nitrate-N, total ammonia nitrogen (TAN) (APHA, 2012) & sulfate-S (Tandon, 2005) and sediment samples were analyzed for sulfate-S (Tandon, 2005) and organic carbon (OC) content (Walkley and Black, 1934) using standard protocols.

2.3. Cultivation and Enumeration of SRB

Cultivation of SRB was done using Postgate medium B with lactate as electron acceptor (Postgate, 1984). Resazurin (0.001 g/L) and thioglycollic acid (0.1 ml/L) were added as oxygen indicator and reducing agent, respectively. pH of the medium was adjusted to 7.5 and sterilized by autoclaving at 121°C for 20 min. A 5% ferrous ammonium sulfate was filter sterilized and added separately at 0.1 ml for every 5.0 ml of the autoclaved medium. Enumeration of viable SRB was done by MPN method using three tube MPN method following FDA-BAM protocol (Sutton, 2010). Headspace oxygen was removed completely by passing oxygen free nitrogen and incubated for the period of two weeks. Formation of black colour precipitate was taken as positive growth and quantification of SRB was done using FDA-BAM table. To study the phylogenetic relationship of SRB, 5 ml of culture from all the highest positive MPN dilutions were transferred to the enrichment media and cultured as mentioned above.

2.4. DNA extraction and PCR amplification.

Genomic DNA from was extracted using a Power Soil DNA Isolation Kit (Himedia laboratories, India). Primer sets targeting *Desulfotomaculum* (Group-1), *Desulfobulbus* (Group-2), *Desulfobacterium* (Group-3), *Desulfobacter* (Group-4), *Desulfococcus–Desulfonema–Desulfosarcina* (Group-5) and *Desulfovibrio–Desulfomicrobium* (Group-6) suprageneric groups of SRB were used (Daly et al, 2000). The amplification reaction mixture contained 1x red dye PCR mix (Ampliqon, Denmark) containing 1mM MgCl₂, 0.4mM dNTPs, and 10 picomoles of each primer. The thermal cycler (Veritii, Applied Biosystems) conditions followed were initial denaturation for 3 min followed by 25 cycles: 94°C for 30 s, 65°C for 30s and 72 °C for 30 s with final elongation for 5 min. Amplified products were electrophoresed on 1.5% agarose gel containing Ethidium bromide in 1x TAE buffer @ 120 V for 30 min and visualized using Gel documentation system.

2.5. Cloning and sequencing

After PCR amplification, unpurified products were purified and cloned with TA (pcrII) cloning vector. The ligation products were introduced into *E.coli* Top10 chemically competent cells, and these cells were plated onto LB medium containing ampicillin and incubated overnight at 37°C. White colonies were randomly selected and screening of positive clones was performed. Clones were analyzed for insert and the purified clones were sequenced using ABI 3730 sequencer.

2.6. Sequence analysis

Sequences containing chimeras were checked with Decipher (Wright et al, 2012). Sequences were aligned using MUSCLE program (Edgar, 2004) and aligned sequences were grouped into Operational Taxonomic Units (OTU) with 97% as the distance cut-off value (Stackerbrandt and Goebel, 1994) using RDP (Ribosomal database project) pipeline tool (Cole, 2003). From each

OTU only one representative sequence was taken to construct phylogenetic analysis. Neighbor joining method was used to construct the phylogenetic tree using, MEGA 5.2.2 (Tamura et al, 2011). Bootstrap analysis was performed with 1000 replicates to verify the reliability of the constructed phylogenetic tree.

2.7. Nucleotide accession number

Representative sequence from each OTU was published in DDBJ database under the accession numbers AB983657-AB983706.

2.8. Statistical data analysis

One- way repeated measures ANOVA was performed to find out the significance in variation of SRB between different places of the pond and days of culture using SPSS 16.0. Pearson correlation analysis was done to find out the relation between physico-chemical parameters of water and soil and SRB population.

3. Results and Discussion

3.1. Changes in Physico- chemical parameters of water and soil

The average water and soil parameters at different days of culture (DOC) in the culture pond are given in Table 1 and the trend of significant parameters at different sampling places in a pond is shown in Fig.1 through 4. pH, DO (ppm), TAN (ppm), total sulfide-S (ppm) and sulfate-S (ppm) content in water during the culture period ranged from 6.78-8.31, 5.43-8.69, 0.055-0.174, and 0.004-0.09 and 1367-1626, respectively. There was no significant difference between days of culture with respect to pond water pH, salinity, turbidity, TAN, nitrite-N, nitrate-N and sulfate-S ($p \geq 0.05$), whereas DO and total sulfide content in pond water and, redox potential and organic carbon content in pond soil differed significantly ($p \leq 0.05$) with the progress of culture period (Table 1).

163 Table 1: Physico-chemical characteristics of water and soil at different days of culture (DOC).
 164 (n=12; mean± SD)

Parameters	DOC 22	DOC 37	DOC 51	DOC 65	DOC 80	Harvest day
Water						
pH	6.99 ^d ±0.02	8.06 ^a ±0.04	7.41 ^c ±0.09	7.48 ^c ±0.30	8.31 ^b ±0.04	6.78 ^e ±0.01
Dissolved oxygen (mg/L)	7.69 ^a ±0.94	6.6 ^d ±1.46	7.52 ^b ±0.63	5.43 ^f ±0.96	6.9 ^c ±2.2	5.85 ^e ±1.36
Turbidity (NTU)	50 ^c ±0.46	65 ^b ±4.59	45 ^d ±2.19	75 ^a ±4.94	72 ^a ±7.45	73 ^a ±1.98
Salinity (ppt)	16 ^b ±1	18 ^a ±1	16 ^b ±0.0	18 ^a ±1	18 ^a ±1	16 ^b ±1
TAN (ppm)	0.096 ^d ±0.007	0.055 ^f ±0.004	0.133 ^c ±0.001	0.084 ^e ±0.002	0.174 ^a ±0.004	0.152 ^b ±0.020
Nitrite-N (ppm)	0.020 ^b ±0.003	0.025 ^b ±0.001	0.012 ^c ±0.001	0.065 ^a ±0.004	0.007 ^c ±0.002	0.078 ^a ±0.00
Nitrate-N (ppm)	0.007 ^e ±0.001	0.027 ^d ±0.0005	0.03 ^c ±0.001	0.036 ^c ±0.001	0.0569 ^b ±0.0011	0.06 ^a ±0.002
Total sulfide-S (ppm)	0.004 ^e ±0.003	0.007 ^e ±0.007	0.028 ^d ±0.011	0.032 ^c ±0.001	0.048 ^b ±0.034	0.089 ^a ±0.029
Sulfate-S (ppm)	1376 ^d ±65	1626 ^a ±43	1518 ^b ±5	1501 ^b ±37	1367 ^d ±32	1416 ^c ±12
Soil						
Organic carbon (%)	0.20 ^e ±0.05	0.27 ^d ±0.08	0.35 ^c ±0.13	0.49 ^a ±0.12	0.34 ^b ±0.18	0.41 ^b ±0.14
Redox potential (mV)	156 ^a ±68.64	61.66 ^b ±29.7	-19.83 ^d ±54	-9.3 ^c ±46	-167 ^f ±114	-123 ^e ±53

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166 Means bearing different superscript in a row are significantly different ($p \leq 0.05$).

167

168 Redox potential in pond soil ranged from 150 to -167 mV during the culture period. Soil redox
 169 potential (E_h) which is the indicator of soil health, was in the positive side 150 & 61.6 mV at the
 170 start of the culture i.e. at DOC 22 and 37 respectively, and then it gradually decreased to negative
 171 values towards the end of the culture (-123 ± 53 mV). This shows that bottom sediment was in
 172 oxidized state during the early phase of culture, which facilitates the aerobic decomposition of
 173 organic matter rather than anaerobic decomposition. During the early phase of culture,

accumulation of organic carbon and availability of substrates will be less, the reason for the E_h values on oxidation scale, whereas E_h values on reduction scale during the later phase of the culture period, is due to the excess availability of substrates obtained from accumulation of unused feed and fecal material. It is evident by the increase in organic carbon content in pond soil from 0.2 % at DOC 22 to 0.41 % at harvest time. Wiyito et al (2016) studied the effect of redox potential in which it was found that negative redox potential generates reduced compounds specifically when the redox value is above -200 mV. It reduces the dissolved oxygen concentration in SWI and increases the sulfide concentration in water column.

3.1.1 Changes in Physico- chemical parameters of water and soil at different places of the pond

When a different place within a pond is taken into account, E_h was highly variable between the three places. Lowest average E_h values of -19.83, -9.3, -167 and -123 mV were observed nearer to SG at DOC 51, 65, 80 and harvest time, respectively (Fig.1). Similar trend of variation was observed in E_h values at the other two sampling points at different days of culture. Similarly, total sulfide content was highly variable at three places with high value at SG (0.116 ppm) followed by PC (0.094 ppm) at harvest time (Fig.2). DO content varied between sampling points within the range of 4.37 to 8.34 mg/L. On the whole, low DO values were found nearer to SG at DOC 65 (4.4 mg/L) and DOC 80 (4.37 mg/L) compared to PC at DOC 65 (5.37 mg/L) and DOC 80 (8.21 mg/L) and WPA at DOC 65 (6.42 mg/l) and DOC 80 (8.31 mg/l) (Fig. 3). Organic carbon content in soil ranged from 0.13- 0.65% throughout the culture period. Fluctuations were observed between sampling points with lowest organic carbon content at WPA (0.13%) during early days of culture and high nearer to SG (0.65%) at DOC 80 (Fig. 2). Overall, there existed a significant difference ($p < 0.05$) in organic carbon, redox potential, dissolved oxygen and sulfide

levels between the three places of the pond. Significantly high organic carbon content (0.49 %) and sulfide (0.05 ppm) and redox potential on reduction scale (-102 mV) and low DO (5.63 ppm) were observed at sluice gate compared to other two sampling places (Table 2).

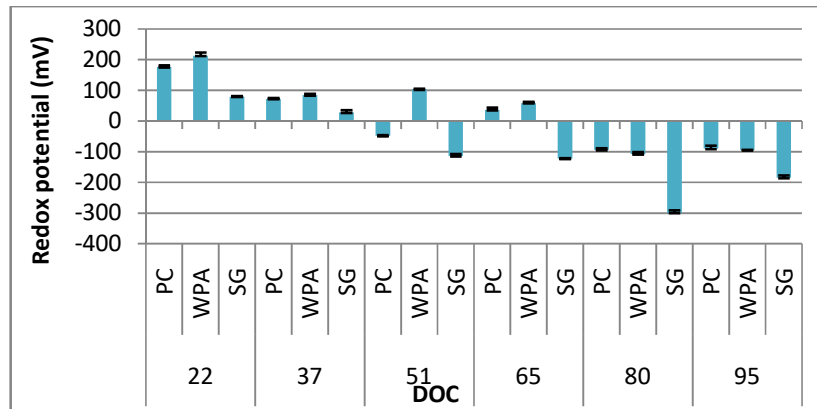


Fig 1: Changes in soil redox potential at three sampling points during culture period. Bars represent means $\pm$ SD

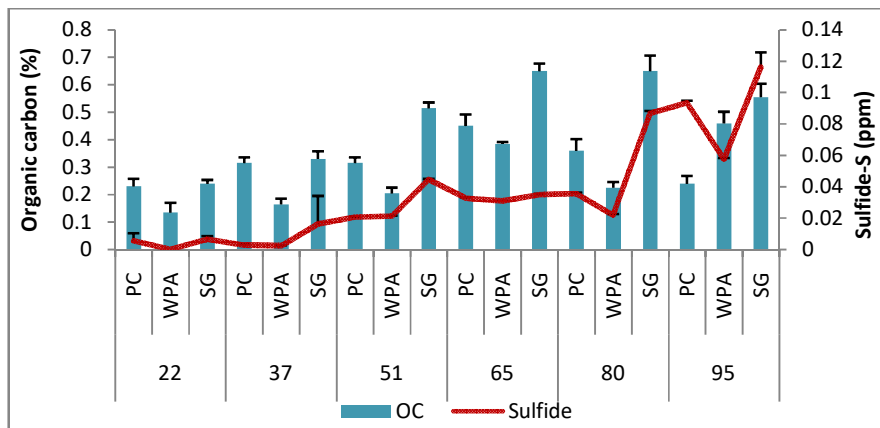


Fig 2: Changes in sulfide content in water and organic carbon content in soil at three sampling points during culture period. Bars represent means $\pm$ SD

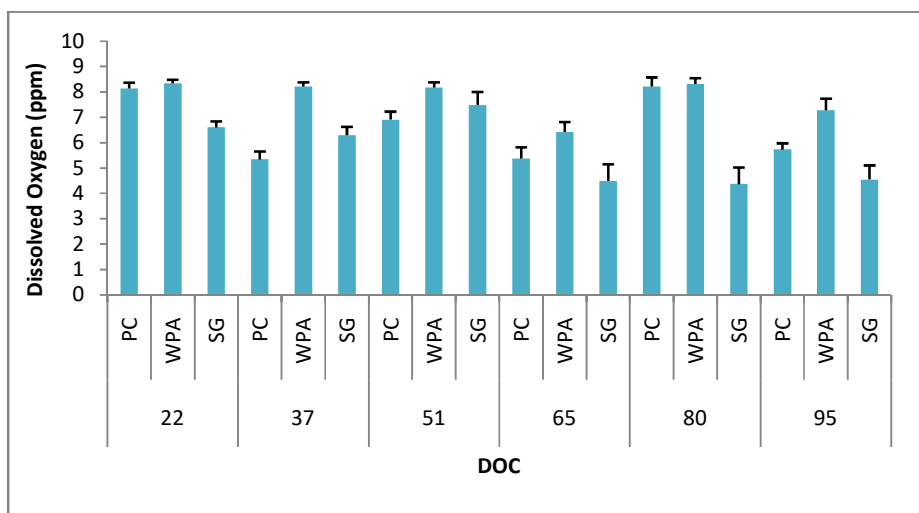


Fig 3: Changes in dissolved oxygen in water at three sampling points during culture period. Bars represent means $\pm$ SD

Table 2: Physico- chemical characteristics of water and soil at different sampling places during culture period (Means with the similar superscript in the same row are not significantly different, $p \geq 0.05$)

Parameter	Sampling place		
	Pond center	Water pumping area	Sluice gate
Water			
DO (ppm)	5.08-9.26 (6.78 ^b $\pm$ 1.51)	6.01-10.59 (8.12 ^a $\pm$ 1.24)	4.02-7.77 (5.63 ^c $\pm$ 1.27)
Sulfide (ppm)	0.002-0.094 (0.032 ^b $\pm$ 0.03)	0-0.058 (0.022 ^c $\pm$ 0.01)	0.003-0.123 (0.051 ^a $\pm$ 0.04)
Soil			
Redox potential *(mV)	180 to -99 (8.5 ^b $\pm$ 99)	221 to -112 (42.61 ^a $\pm$ 115)	81 to -305 (-102 ^c $\pm$ 130)
Organic Carbon (%)	0.21-0.48 (0.31 ^b $\pm$ 0.07)	0.11-0.49 (0.26 ^c $\pm$ 0.12)	0.23-0.69 (0.49 ^a $\pm$ 0.16)

* Range is given for redox potential as the values were from oxidation scale (+ve) to reduction scale (-ve).

3.1.2 Changes in Physico- chemical parameters along the depth of pond

In case of vertical distribution, OC was fluctuating along the depth and there was no particular trend observed throughout the culture period. Comparing all the cores, OC content (%) was found to be high in SWI (0.5cm) at DOC 22 (0.26 ± 0.03) and at harvest time (0.55 ± 0.03), whereas at DOC 51 high OC was found in 1.5 cm cores (0.61 ± 0.014) compared to 0.26 ± 0.04 in SWI (Fig. 4). Likewise sulfate content in soil was also highly variable along the depth, throughout the culture period. Maximum sulfate concentration (mg/L) was observed in 5.0 cm core at DOC 22 (1218 ± 29.69), 7.5 cm layer at DOC 51 (1733 ± 32.10) & 1.5 cm layer at harvest time (1354 ± 16.37) (Fig.4).

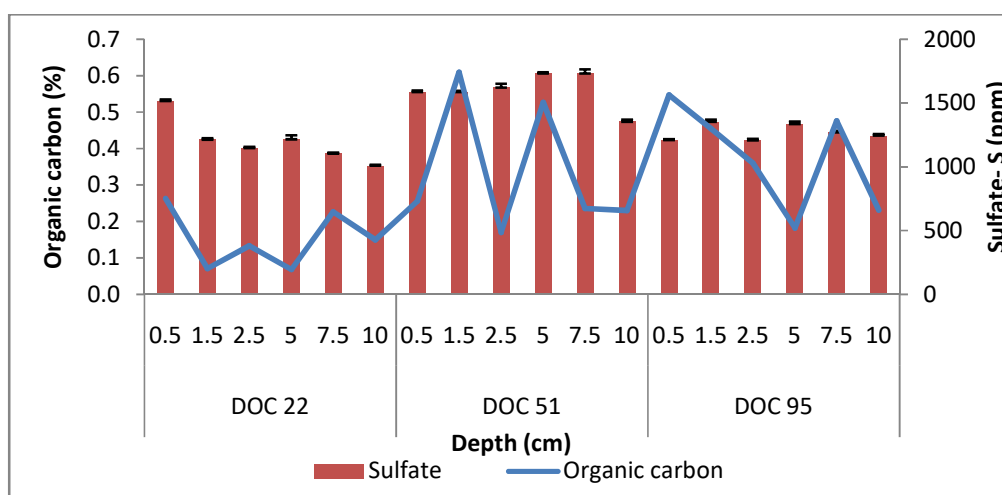


Fig 4: Variation of sulfate and organic carbon content in soil along the depth of pond during the culture period. Bars represent means $\pm$ SD

3.2. Enumeration of SRB

3.2.1 Horizontal distribution of SRB at different places of the pond

Distribution of SRB varied between different places of the pond, high numbers registered at SG followed by CP and WPA (Fig. 5). Throughout the culture period, number of SRB was seen fluctuating with no constant trend at different DOC nearer to WPA and PC, whereas the numbers

found to be high at SG (>1100 MPN/g) on DOC 51 and it remained constant thereafter. A significant difference ($p \leq 0.05$) was found in means of SRB numbers between three places of the pond and between the days of culture. Post hoc test revealed significantly high SRB population at SG (874 MPN/g), followed by PC (272 MPN/g) and WPA (99 MPN/g) (Fig.6). No significant difference was observed with SRB numbers between DOC 51 and 65 & 80 and 95 ($p \geq 0.05$).

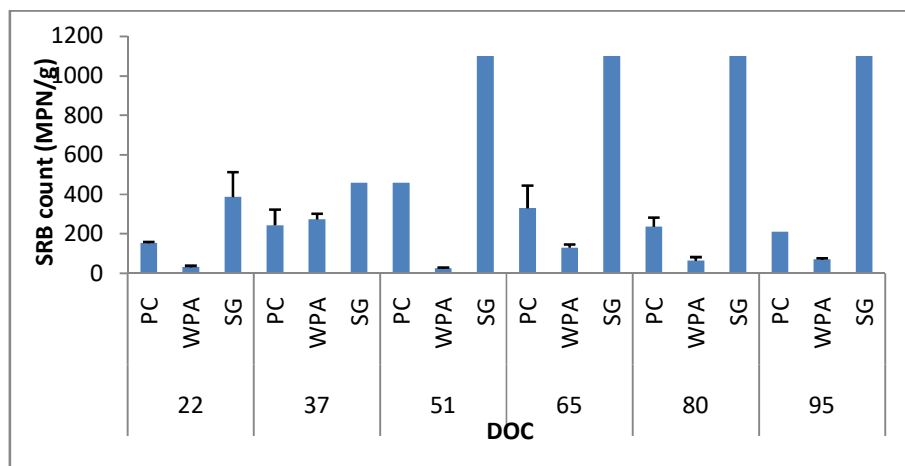


Fig 5: Horizontal distribution of SRB at different sampling points during culture period. Bars represent means $\pm$ SD

3.2.2: Vertical distribution of SRB along the depth of pond

The average number of SRB at DOC 22, 51 and harvest time was 30, 431, 684 MPN/g, respectively (Fig.7). On DOC 22 from SWI to 10 cm the number of SRB was 53 MPN/g at SWI and decreased up to 0.5 cm (30 MPN/g) and then remained constant throughout 10 cm cores. At DOC 51, number of SRB was higher in 0.5 and 1.5 cm cores (>1100 MPN/g), decreased from 2.5 to 5.0 cm (150 MPN/g) and further decreased to 30 MPN/g from 7.5 to 10 cm cores. In contrast, at DOC 95, number of SRB was found to be constant (>1100 MPN/g) in 0.5- 2.5 cm cores and then decreased to 290 MPN/g in the remaining cores. A significant difference was

found between the numbers of SRB ($p \leq 0.05$) along the depth of the pond except in 0.5 cm and 1.5 cm cores (Fig.8).

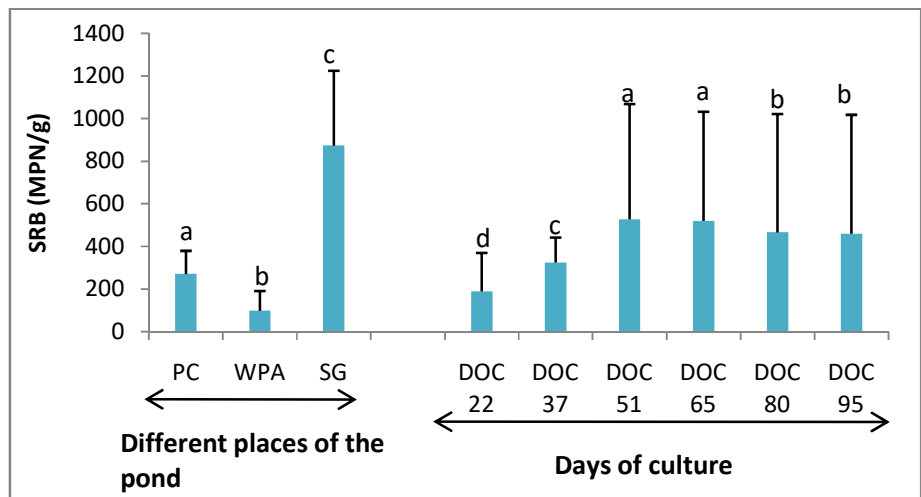


Fig 6. SRB population at different sampling places and DOC (MPN/g) in three places of the pond and different DOC (Means with the same letter are not significantly different $p \geq 0.05$. Bars represent means $\pm$ SD)

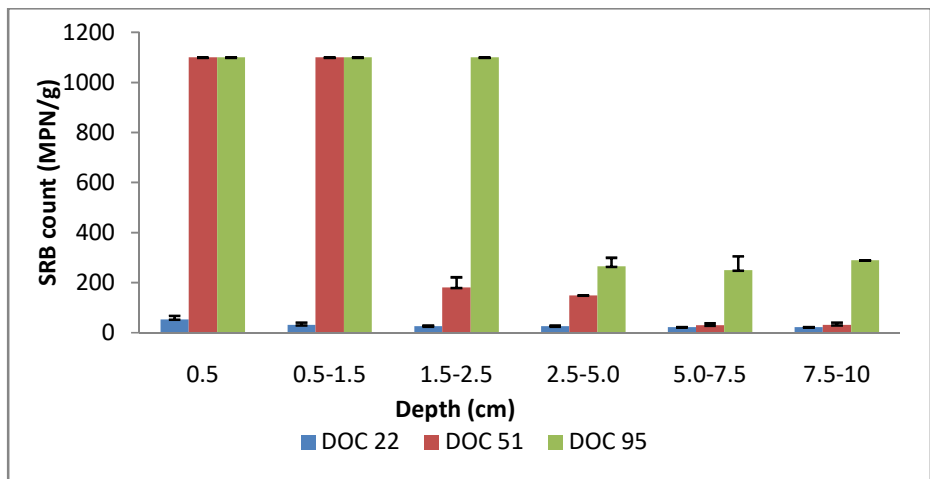


Fig 7: Vertical distribution of SRB (MPN/g) along the depth of pond during culture period. Bars represent means $\pm$ SD

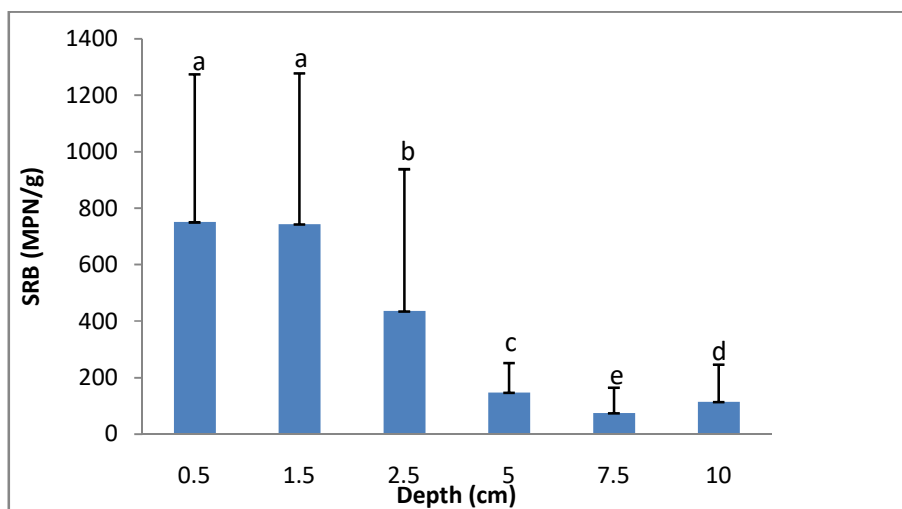


Fig 8: SRB population (MPN/g) along the depth of pond (Means with the same letter are not significantly $p \geq 0.05$). Bars represent means $\pm$ SD

On the whole, number of SRB was found to be high nearer to SG followed by PC and WPA and their distribution along the depth is found to be decreasing with high numbers at SWI. Similar kind of result, with maximum number of SRB in top 3cm layer ($6.6 \pm 1.0 \times 10^8$ cells cm^{-3}) and then decreasing numbers below 3cm layer ($1.7 \pm 0.8 \times 10^6$ cells cm^{-3}) was observed in Wadden Sea sediment (Brossa et al, 2002). Urakawa et al. (2000) studied the variation of microbial communities along the depth in marine sediments. They observed large number of SRB in surface layers compared to the deeper depth because of organic matter availability. Generally SRB is expected to be high in deeper sediments due to anaerobic condition in lakes and marine waters. During shrimp culture, unused feeds and waste materials will be deposited on surface sediments. In the presence of sulfate, these complex macromolecules will be utilized by SRB whereas, in absence of sulfate, SRB ferment organic acids through fermentative bacteria by which fermentation compounds like lactate, acetate and propionate will be produced. Holmer and Storkholm (2001) observed high sulfate reduction rate in surface layers of fish farmed sediments,

where accumulation of organic matter was high. Thus, SWI provides ideal condition for the anaerobic dissimilatory sulfate reduction and fermentative growth of SRB. Presence of sulfide levels of 0.004, 0.028 and 0.09 ppm during DOC 22, 51 and harvest time, respectively (Fig. 2) indicates the effective sulfide production in the pond sediment.

3.3 Correlation between SRB and Physico-chemical parameters of pond water and soil

Significant negative correlation was found between SRB numbers and dissolved oxygen ($r = -0.633$) and redox potential values from oxidation to reduction scale ($r = -0.672$). A significant positive correlation was found between SRB numbers and organic carbon content ($r = 0.812$) and sulfide in water ($r = 0.525$). In case of vertical distribution, significant positive correlation was found between organic carbon in soil and SRB ($r = 0.651$) and a weak correlation ($r = 0.109$) was observed with sulfate. Many studies have reported that organic carbon as a main limiting factor in shaping the abundance and distribution of SRB. Kondo et al (2012) studied SRB from Japanese fish farm sediments with different level of organic enrichment in which fish pond with high organic pollution found to be more diverse in SRB. Kawahara et al (2009) observed that organic enrichment influences the bacterial communities and clones related to SRB were found to be in higher frequency from organically enriched surface sediments (0-4 cm) of fish farm in Wakasa Bay of Japan. Santander et al (2015) studied the bacterial communities in milk fish cages from Philippines in which they observed many sulfate reducing bacteria and other spore forming genera. They further explained that organic load derived from higher fish feed and its left over constituents, created reduced environment through which anaerobic bacteria flourish. It is evident from this study that organic matter accumulated in the form of unused feed and faecal materials play a major role in sulfate reduction.

3.4. PCR amplification

304 DNA was isolated from highest positive (>1100/g) MPN dilutions and amplification of partial
305 fragments of 16S-rDNA genes using group specific primer yielded expected base pair in all the
306 enrichment cultures for group 5 & 6. No bands were found in groups 1, 2, 3 and 4. More than
307 100 clones in group 5 & 6 were screened for an insert. Twenty seven clones in group 5 and 43
308 clones in group 6 were positive. All the 70 clones were sequenced in which eight sequences were
309 found to be chimeric and removed from further analysis. The remaining clones were grouped
310 into 50 OTU's (16 in group 5 & 34 in group 6) using 0.03% as distance cutoff value.

311 **3.5. Phylogenetic analysis**

312 Neighbor joining method was performed to visualize the phylogenetic relationship of clones
313 isolated from shrimp farm sediments, with clones isolated from other habitats available in NCBI
314 database with *Desulfuromonas acetoxidans* (AY188891) as outgroup. Group 5 (*Desulfococcus*–
315 *Desulfonema*–*Desulfosarcina*) formed five clusters comprising *Desulfococcus*, *Desulfonema*,
316 *Desulfosarcina*, *Desulfatiferula* and *Desulfobotulus* (Fig. 9).

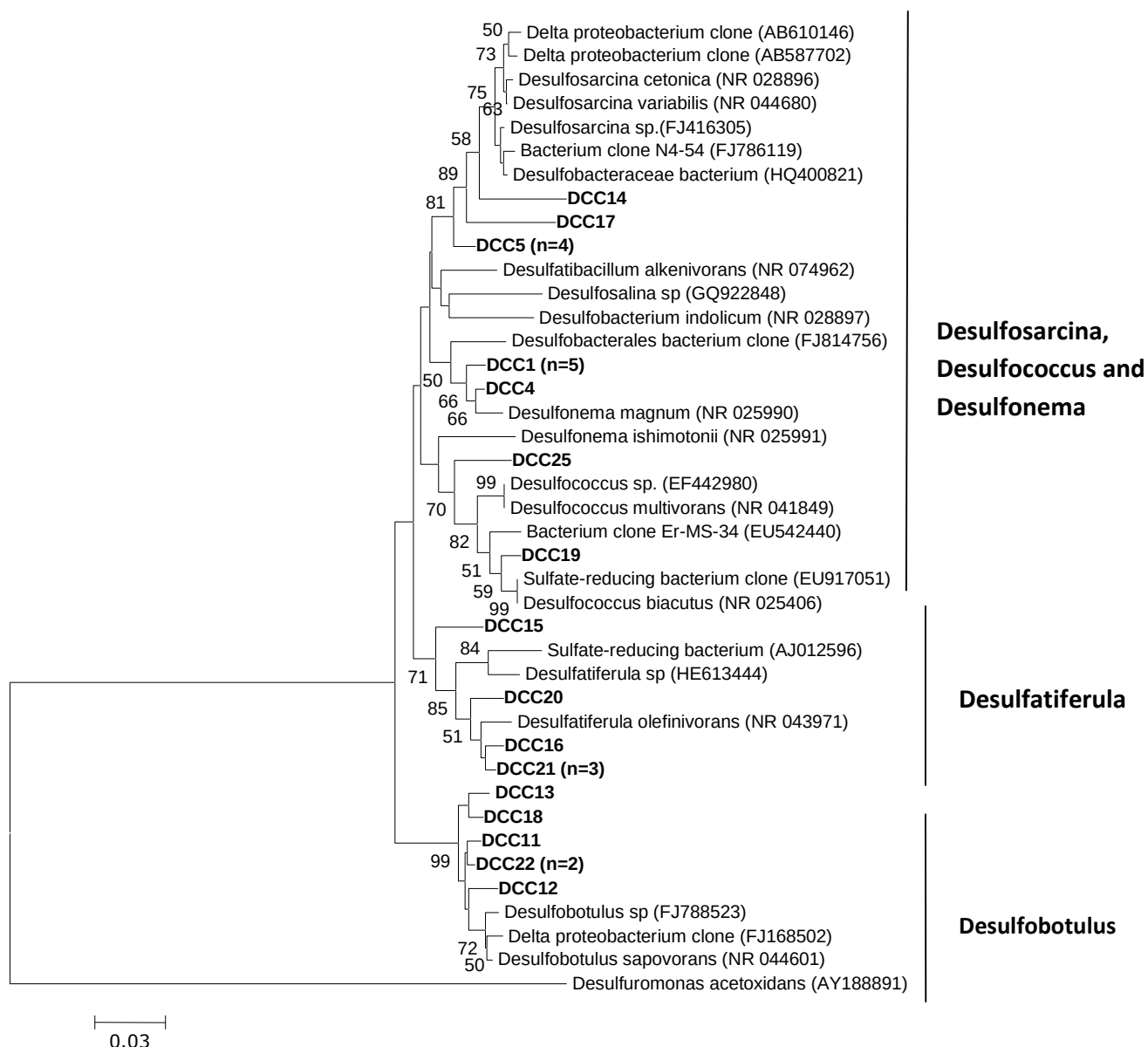


Fig 9: Phylogenetic tree showing representative 16SrRNA gene sequences related to Group-5 isolated from shrimp cultured sediments (shown in bold). Number of clone sequenced in each phylotype is given in the parentheses.

323 Cluster 1 had five phylotypes (DCC12, DCC18, DCC11, DCC22 (n=2), DCC13) which shared
 324 phylogenetic relationship with *Desulfobotulus sapovarans* (NR-044601) isolated from hyper
 325 saline sodalake sediment. Cluster 2 consist of four phylotypes (DCC21 (n=3), DCC16, DCC20,
 326 DCC15) related to *Desulfotatiferula olefinivorans* (NR_043971) isolated from brackishwater
 327 sediment of oil refinery which can utilize only sulfate as electron acceptor and C₁₄-C₂₄ fatty acids
 328 as electron donor (Laureau et al, 2007). Cluster 3 had two phylotypes DCC19 and DCC25 that
 329 formed monophyletic relationship with *Desulfococcus biacutus* (NR_025406) and environmental
 330 clone (EU917051) recovered from crude oil gathering system of China (Liu et al, 2008). DCC25
 331 formed a separate lineage within *Desulfococcus* group. Cluster 4 had six clones (DCC1 (n=5)
 332 and DCC4) which is related to *Desulfonema magnum* (NR_025990) isolated from anaerobic mud
 333 of sea water lagoon. This is one of the gliding filamentous sulfate reducing bacteria in
 334 deltaproteobacter, which can oxidize acetate and aliphatic acids (Fukui et al, 1999). Cluster 5 had
 335 three phylotypes (DCC14, DCC17 and DCC5 (n=4) which formed a separate lineage from
 336 bacterial clone (FJ786119) isolated from shrimp farming sediment within *Desulfosarcina* sp.,
 337 (FJ416305).
 338 Group 6 formed three distinct clusters comprising *Desulfomicrobium* sp., *Desulfovibrio* sp.,
 339 *Clostridium* sp., (Fig.10) in which cluster 1 with 14 phylotypes namely DSV7, 14, 5, 4, 10, 3, 1
 340 (n=2), 8, 15, 12 (n=2), 6 and 24 formed a separate lineage within *Desulfomicrobium* sp., and
 341 clones DSV2 and DSV9 shared its relationship with *Desulfomicrobium* sp., (AY548759).
 342 Cluster 2 had 14 phylotypes namely DSV30, DSV29 & DSV31 formed a monophyletic clade
 343 with *Desulfovibrio* sp., (AJ251630), *Desulfovibrio inopinatus* (NR025038) and *Desulfovibrio*
 344 *desulfuricans* (NR074858) respectively. DSV27, 35 and DSV21 formed monophyletic clade with
 345 *Desulfovibrio piezophilus* (NR_102518), *Desulfovibrio* sp., (KF733439) and *Desulfovibrio*

346 *salexigens* (NR102801) respectively. Other clones (DSV28, 25, 22, 17, 19, 18, 16, and 26)
347 formed independent cluster within *Desulfovibrio* group.



348
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Fig 10: Phylogenetic tree showing representative 16SrRNA gene sequences related to Group 6 isolated from shrimp cultured sediments (shown in bold). Number of clone sequenced in each phylotype is given in the parentheses.

Desulfovibrio spp., which belongs to deltaproteobacter, is dominant in organic rich marine habitats. In the presence of sulfate, fermentation products like lactate, malate and fumarate can be used as electron donors and even in the absence of sulfate, it can utilize pyruvate and formate (Widdel and Bak, 1992). Saas et al (1997) studied the vertical distribution of SRB in the oligotrophic lake sediments, in which many strains related to *Desulfovibrio* sp., and *Desulfomicrobium* sp., were found in oxic-anoxic layers and these bacteria, being catalase positive organism, shows higher oxygen tolerance capacity and they are well adapted to live near sediment water interface. *Desulfovibrio* has also been isolated commonly from corals affected by Black Band disease (Cooney et al, 2002 & Bourne et al, 2011). Around eleven clones related to *Desulfovibrio desulfuricans* (with 97% similarity) were isolated from diseased corals in Northern Red sea (Barneah et al, 2007). Interestingly two clones DSV17 & DSV19 isolated in this study were similar to *Desulfovibrio* sp., isolated from coral montipora in Japan.

Cluster 3 had six clones (DSV32, 36, 34, 23, 20 and 33) in which DSV32 and DSV23 formed monophyletic cluster with *Clostridium* sp., (AB470961) and *Clostridium aestuarii* (DQ126679) isolated from tidal flat sediment which is closely related to type species *Clostridium butyricum*. DSV36 & DSV34 formed independent lineage within family Clostridiales, a group of spore forming bacteria under phylum Firmicutes which have the potential to switch to an energy conserving metabolism with low sulfate or even with no sulfate (Leloup et al, 2009). Tamminen et al, (2011) also observed clones similar to Clostridia from Rainbow trout fish farms with black

373 muddy sediment near Baltic Sea. It is further explained that clones related to Clostridia, more
374 likely to have originated from fish gut micro biota. Moreover, this is possible because many of
375 the soil probiotics used in aquaculture contains *Clostridium butyricum* as one of the essential
376 component.

377 It is interesting to note that Clones related to group *Desulfotomaculum*(Group-1), *Desulfobulbus*
378 (Group-2), *Desulfobacterium*(Group-3), *Desulfobacter* (Group-4) which are reported to have
379 isolated from both marine and fresh water sediments are completely absent in this study. This is
380 possibly because of substrate availability and organic enrichment in the shrimp culture
381 sediments. Kawahara et al, (2009) suggested that composition and activity of SRB is influenced
382 by amount of organic enrichment in aquaculture. Castine et al, (2009) studied the effect of
383 organic load in tropical finfish cage farm in which, they found genus related to *Desulfovibrio*,
384 *Desulfobacter*, *Desulfococcus*, *Desulfonema* in higher abundance. They further suggested that
385 anaerobic zones created by organic matter load plays a key role in selection of bacteria involved
386 in sulfate reduction. Understanding the microbial interaction in pond bottom is necessary in
387 shrimp aquaculture because more than any other aquatic species, shrimp dwell on the bottom and
388 ingest pond-bottom soil (Boyd, 1989). Avnimelech (1995) estimated that 50% of the pond
389 bottom in shrimp culture is covered by reduced sediment with a typical black color and smell of
390 hydrogen sulfide which affects the shrimp growth, activity and health.

391 Abraham et al, (2004) studied the population of SRB in shrimp culture systems of India, in
392 which significant difference was found in population of SRB in semi intensive and traditional
393 ponds because of varying degree of substrate availability and anaerobic processes. Suplee and
394 Cotner (1996) studied sulfide fluxes in shrimp ponds over a period of 17 months during which
395 they found organic matter availability and its reactivity was the predominant factor in

determining the sulfide fluxes in shrimp pond. They also compared sulfate reduction in old and newly constructed shrimp ponds, in which significant difference was observed between two ponds because of higher organic matter deposition in old ponds over the years.

Farm management practices which includes stocking, feeding, and inputs application during the culture period plays a key role in shaping up the microbial community in pond bottom. Generally in shrimp culture sediments, as the days of culture increases, pond bottom becomes more and more reduced due to application of feed rich in protein, accumulation of nutrients, unused feed and fecal materials. This provides suitable environment for anaerobic microorganism to flourish and in addition to this, brackishwater shrimp ponds contain high level of sulfate in sea water which provides enormous scope for sulfate reduction to take place. Moreover, in organic matter rich sediments, methanogenic archaea compete with SRB for common substrates like acetate, H_2 and other organic compounds. Since, SRB has more affinity towards these common substrates, they generally out compete methanogens which further favours the process of sulfate reduction (Mitter, 2010).

Despite high saline sediments having huge scope for sulfate reduction; there are studies which demonstrated sulfide production in low saline aquatic sediments (Holmer and Storkholm, 1996 & Pallud and Cappellen, 2006). Liu et al, (2009) quantified the sulfate reducing prokaryotes in paddy sediments. They observed sulfate reduction in rice paddy sediments equal to marine sediments and it is further mentioned that in low sulfate environments, sulfate reduction happens via fermentative growth of SRB. This indicates that sulfate reduction will be an equally important process even in low saline shrimp culture sediments as the organic matter accumulation and anaerobic condition are same vertically from SWI.

Conclusion

In this study, we evaluated the distribution of SRB horizontally and vertically and its phylogenetic diversity brackishwater shrimp culture ponds. In conclusion, SRB was higher nearer to SG followed by PC and WPA and at sediment water interface compared to deeper depths. Factors like dissolved oxygen in water, redox potential and organic carbon content in soil had a key role in sulfate reduction and were correlated with SRB numbers. Genera like *Desulfonema*, *Desulfosarcina*, *Desulfatibacillum*, *Desulfobotulus*, *Desulfomicrobium*, *Clostridium*, *Desulfococcus* and *Desulfovibrio* were found to be present in brackishwater shrimp culture sediments. Future investigations with regard to the role of various organic matter sources on sulfate reducing bacteria communities will certainly help in understanding the process of sulfate reduction and its mitigation in shrimp culture sediments

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