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IDENTIFICATION AND OCCURRENCE OF *Vibrio parahaemolyticus* IN SHRIMP PONDS OF SELECTED PONDS IN PONNERI, TIRUVALLUR DISTRICT

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Short Research Article

ABSTRACT

The present study aimed to monitor the occurrence of *Vibrio parahaemolyticus*, an opportunistic prawn pathogen, in two white-leg shrimp *Litopenaeus vannamei* culture ponds (from stocking to harvesting period), each located in the villages Pullikulam and Kumarasirulapakkam of Thiruvallur District. They were selectively isolated, counted using serial dilution and confirmed by 16s rDNA sequencing. Our study showed that *V. parahaemolyticus* occupied one tenth of the total microbial load in the ponds. The count ranged from 1000 to 1600 CFU/ml in water at the time of stocking and varied with time period. The sediment always harbour more bacteria than water and the number would reach up to 35×10^4 CFU/g in the harvesting period. Both the ponds showed a steep increase on the 30^{th} day of stocking and then there is a gradual decrease or increase in the water parameter. The occurrence of the bacteria was found to depend on the water and soil quality parameters that vary with timelines. The study showed that the bacterial count increased with the culture periods in both the ponds. This would serve to be an indirect evaluation for the health assessment of the shrimps and raises the need for continuous validation on their loads.

Keywords: *Vibrio parahaemolyticus*; *Litopenaeus vannamei*; microbial load; *Vibrio* content; water quality parameters; soil quality parameters.

1. INTRODUCTION

Shrimp farming is a multi-billion dollar industry which contributes a major income to many Asian countries. Until 2009, India had termed its productivity synonymously for the tiger shrimp (*Penaeus monodon*) but the incidence of White spot syndrome virus (WSSV) lead to the stagnation of tiger shrimp farming since 1995. Hence, there was a shift in the production species from *P. monodon* to *Litopenaeus vannamei* which had replaced 90% of the world's shrimp productivity. India is the second largest country in aquaculture production in the World including white-leg shrimp, *L. vannamei* culture [1]. Due to the ability of the species to adopt in lower-salinity waters, the Pacific white shrimp has become the candidate of choice in Indian exportable shrimp culture [2]. In India, Andhra Pradesh predominately

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contributes for the export value followed by Tamil Nadu [3].

Water and soil quality is one of the most important prerequisite for a sustainable aquaculture, including shrimps [4]. The water and soil quality parameters play a vital role in growth and survival of the cultured shrimps which could be achieved only at optimal conditions. Thus, the important parameters of water and soil have been studied selectively from time to time due to the daily environmental changes by various monitoring analysis to achieve their optimal level for higher profit. With the development of shrimp culture practice from traditional form to modern intensive culture practice, the complexity of diseases has been equally magnified in India. Shrimp aquaculture industry has experienced severe setbacks due to devastating diseases [3]. The microbial population also has an equal contribution as water and soil parameters to maintain optimal growth and nutrient values.

Shrimp culture routinely faces a hardship from the abundance of *Vibrio* spp., due to contamination of moulted shells from the dead ones and nutrient loading. *Vibrio* is recognized as a major causative agent for wounding and blood infections in shrimp culture [5]. They have been shown to turn into opportunistic pathogen while there is a stressed environmental condition on culture ponds. Thus, the risk assessment also depends on the water and soil quality parameters of culturing ponds. Therefore, the present study is an attempt to identify the correlation of the *Vibrio* spp. load with that of the water and soil quality parameters in selected shrimp culture ponds. This study in one way depicts if the quality parameters influences the *Vibrio* load.

2. MATERIALS AND METHODS

2.1 Description of Study Site

For the present study, two inland culture ponds (1 acre each) growing *L. vannamei* were selected from two villages *viz*., Pullikulam and Kumarasirulapakkam located in Anuppampattu (AP; 13.3043°N 80.2310°E) and Devadhanam (DN; 13° 18' 34N 80° 14' 41E) Revenue town panchayats, respectively, of Ponneri taluk, Tiruvallur district with approximately 5 km distance between them. The ponds mainly rely on the water sources fed from their grounds. In these ponds, the shrimps are given pelleted feed thrice a day.

2.2 Sample Collection

Water and sediment samples were collected aseptically from the ponds in sterile screw-capped polythene containers and tight plastic bags, respectively, brought to the laboratory for further analysis and microbial screening. The samples were properly processed for further studies within three hours after collection.

2.3 Analysis of Physicochemical Parameters

Water temperature, pH and salinity were recorded at the site itself using a Celsius thermometer, portable pH meter and portable refractometer, respectively. A total of 14 water parameters such as pH, turbidty, total hardness, total alkalinity, nitrate, total suspended solids, chemical oxygen demand (COD), sulphide, dissolved oxygen (DO), ammonia, salinity, total nitrogen, biological oxygen demand (BOD) and redox potential and 8 soil parameters such as pH, bulk density, water holding capacity, lime requirement, redox potential, sulphide, available nitrogen and phosphorous were analysed [6]. All the water and soil parameters were estimated following standard methods from APHA [7].

2.4 Microbial Screening

The total bacterial count in water and sediment samples was done using Brain heart infusion (BHI) agar by serial dilution procedure. The *Vibrio* spp. were selectively isolated using thiosulfate citrate bile salts sucrose (TCBS) agar adopting manufacturer's instructions (Himedia, India). The pure isolated blue-green colonies were inoculated in nutrient broth and DNA was extracted from the *Vibrio* isolates using standard protocol (QIAamp DNA mini kit, Qiagen Pvt. Ltd., Germany). 5'CCGAATTCGTCGACAACAGAGTTTGATCAT GGCTCAG3' was used as forward primer and16S rDNA analysis was done following the procedure described by Weisburg et al. [8].

2.5 Statistical Analysis

Statistical significance of associations (dependence) of estimated *Vibrio* spp., content was tested using correlation analysis. Tukey post hoc test was applied to determine which groups and days were statistically different. A confidence interval at the 95% level ($P < 0.05$) was considered in all cases. Pearson's correlation was used to the correlation between bacterial counts in water and sediments. Relevant theoretical inputs for statistical analyses were adopted from Zar [9].

3. RESULTS AND DISCUSSION

The water samples were collected from two ponds (Pullikulam and Kumarasirulapakkam villages) from Thiruvallur district for the study. The samples were collected aseptically with extreme care and were brought to the laboratory.

Table 1. pH and temperature recorded at the time collection (on-site)

S. no.	Pond Details	Time of collection	Temperature (°C)
1.	Anuppampattu (AP)	Morning	30.5±0.5
2.	Devadhanam (DN)	Morning	29.8±0

The value was represented as Average ± Standard Error (n=20)

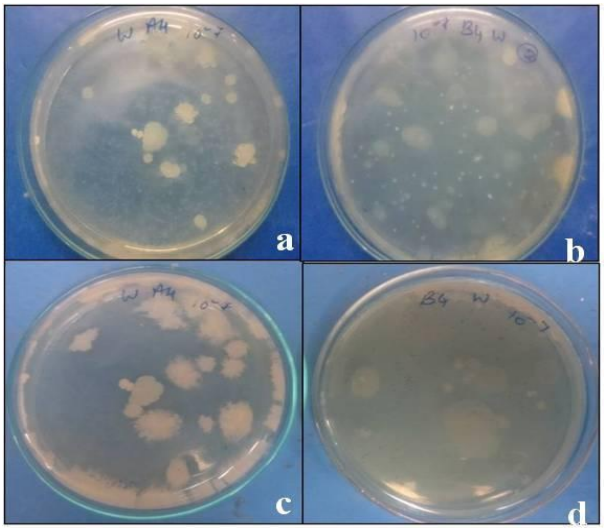

Fig. 1(a-d). Total Bacterial load from two shrimp ponds. BHI agarplates for enumerating the total bacterial count in water and soil samples (a and c) AP sample, (b and c) DN sample

The pH and temperature of the samples was recorded at the time of sampling (Table 1). The water and sediment samples from both the ponds were evaluated for their total microbial content and the occurrence of the *Vibrio* spp. (Fig. 1a-d). Serial dilution and plating on BHI agar revealed that the total microbial content outnumbered at the time of stocking with 100–160 CFU/ml in water (Fig. 2). The sediment samples of pond AP contained approximately tenfold increased (1700–1900 CFU/g) bacterial colonies than the water samples. The number slightly increased with the advancement of time as the sediments always harbour more bacteria than water and the number reached up to 35×10^4 CFU/g on harvesting period.

The sediments from Pond DN contained nearly the same count as pond AP and followed a similar pattern of distribution throughout the culturing period. Both the ponds showed a steep increase on 30th day of stocking and then gradual increase with time based on other parameters. Angela et al. [10] also found a similar bacterial distribution that varied with different time intervals in *L. vannamei* culturing ponds. The samples were serially diluted and inoculated in BHI agar for 24 hours at 37°C. The colony forming units were expressed as Average ± Standard Error (*n*=20).

TCBS agar was used for selective isolation of *Vibrio* spp. from both the ponds. The samples were serially diluted and inoculated in TCBS agar for 24 hours at 37°C. The colony forming units were expressed as Average ± Standard Error (*n*=20). The colony number contained in both the samples was tenfold lower than the total bacterial load. Anuppampattu pond showed higher count ($150–170\times10^2$ CFU) than that of Devadanam ($120–150\times10^2$ CFU) on stocking but with a sharp reduction at 60 days. Generally on both ponds, *Vibrio* spp. was found to be lower and its count did not exceed 120–150 CFU/g and 40–60 CFU/ml in sediment and water samples, respectively (Fig. 3).

A steep increase was recorded on the 30th day after stocking in AP samples but it reduced and normalized on subsequent days, due to treatments. Ganesh et al. [11] showed similar *Vibrio* spp. pattern on the 25th day of stocking in geographically likewise (near to coastal region) shrimp ponds of Cuddalore district. The water and sediment samples from each pond were collected and serially diluted and plated in TCBS agar. The isolated *Vibrio* colonies were identified using 16s rRNA sequences.

The DNA from the blue-green colonies isolated from the TCBS plates were identified as *V. parahaemolyticus* HpB4 using 16s rRNA sequencing (Fig. 4, Table 2). The length of the PCR product was 1500 bp (yet to be deposited in Genbank). The results

retrieved were 16s rRNA gene sequences of *Vibrio* spp., with 100% similarity to *V. parahaemolyticus* strain 22702 (GenBank accession number: EF203421.1) and 99.4% similarity to *V. parahaemolyticus* strain ATCC 17802 (GenBank accession number: CP014046.2). The water and sediment parameters from both the samples were also evaluated to facilitate the assessing of *Vibrio* spp., distribution. *V. parahaemolyticus* is a gram-negative halophilic bacterium distributed in shrimp ponds predominately and may lead to severe food-borne gastroenteritis [11]. The *V. parahaemolyticus* distribution predominately vary with water and sediment parameters of the individual ponds.

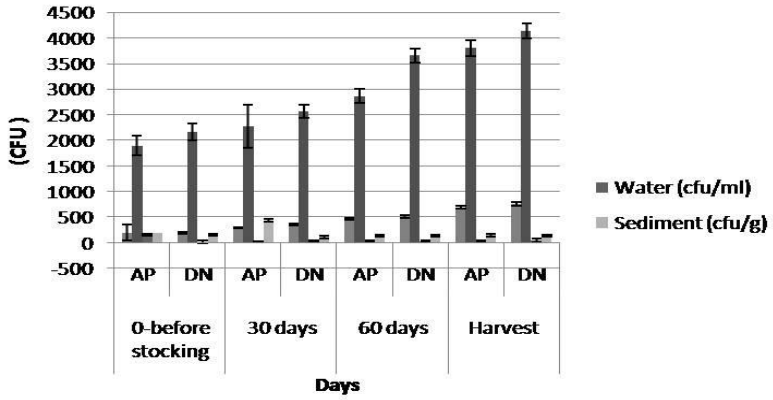

Fig. 2. Total Bacterial load in water and sediment samples from AP and DN culture ponds

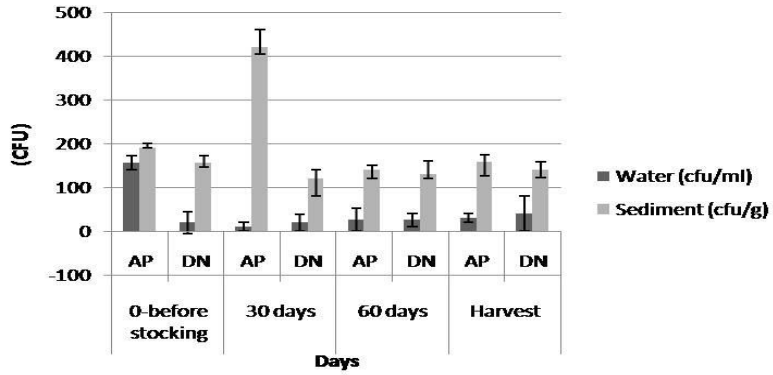

Fig. 3. *Vibrio* spp., load in water and sediment samples from AP and DN culture ponds

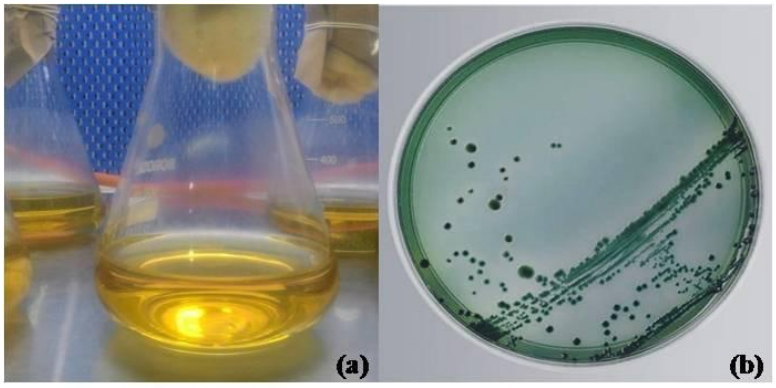

Fig. 4. Enumeration and confirmation of *Vibrio* spp. (a) Culture broth of the isolated *Vibrio* spp., (b) Characteristic (blue-green colony) growth of *V. parahamolytius* in TCB agar

Table 2. Consensus sequences of amplified 16S rDNA gene of isolated *V. parahaemolyticus*

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGAAA
CGAGTTATCTGAACCTTCGGGGAACGWTAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTA
GGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGG
GCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGT
GAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGA
ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAG
CCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAG
GAAGGCGGGKTMGTTAATAGCGTMWTCGTTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTC
CGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAAC
TGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATC
TGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGT
GGCCTTGAGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTTGGGAGTACGGTCGCAAG
ATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGC
AACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCG
GGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCGAGTAATGTCGGGAACTCCAGGGAGACTGCC
GGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTAC
ACACGTGCTACAATGGCGCATACAGAGGGCRGCCAACTTGCGAAAGTGAGCGAATCCCAAAAAG
TGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTG
GATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGT
GGGCTGCAAAAGAAGTAGGTAGTTTAACCTTCGGGGGGACGCTTACCACTTTGTGGTTCATGACT
GGGGTGAAGTC

The analytical results of different water and soil quality parameters were represented in Tables 3 and 4. Water quality plays a vital in shrimp culture to attain the maximum commercial profit. Shrimps are more prone to fluctuations in water parameters. In the present study, we analysed different parameters such as turbidity, total hardness, alkalinity, nitrate etc. at different time lines for ensuring the larval health during the culture timelines. The optimum BOD level for shrimp culture should be between 8 and 11 mg/L. The BOD level of selected samples was found to be similar to the standard value. Dissolved oxygen (DO) is responsible for vital processes such as growth, survival, distribution, behaviour and physiology in shrimp culture and other type of aquacultures. Our results indicated that DO of the sampling sites fall between the standard limits. The water samples were collected at different time intervals and analysed using standard methods. The results were expressed as Average ± Standard Error (*n*=20).

The sediment quality plays an important role in maximum productivity and it is the principal moderator for giving nutrient-rich and healthy environment to the shrimps. In the present study, sediment qualities such as its pH, available nitrogen and phosphorus, bulk density and water holding capacity were within the limits. These results showed that optimal conditions could be a cause for the decrease or threshold maintenance of bacterial load in respective ponds. The sediment samples were collected at different time intervals and analysed using standard methods. The results were expressed as Average ± Standard Error (*n*=20).

The correlation analyses between microbial loads in water, sediment and *Vibrio* spp., distribution in respective samples revealed that there was a significant positive correlation among them ($P < .01$). Analysis made using ANOVA showed that the observed differences between them were statistically significant ($P < .01$) as represented in Table 5. Similarly, Lekshmy et al. [12] reported a correlation between the *V. cholera* and water and sediment parameters of shrimp pond from Kerala coastal region. Our results were found to be concordant with their study.

Fernanda et al. [13] reported that the sediment's microbial load and diversity of *L. vannamei* rearing ponds directly mimicked the shrimp's intestinal microbial flora. They also showed that bursting microbial load may lead to the early development of AHPND/EMS disease in shrimps. In our study, though we found a steep increase in microbial load at the time of stocking and it could be overcome by regular microbial maintenance to keep the *Vibrio* spp., content at the threshold level as mentioned from previous studies.

Table 3. Physico-chemical parameters of water samples taken from AP and DN ponds

S. no.	Water parameters	Units	0-before stocking		30 days		60 days		Harvest	
			Pond AP	Pond DN	Pond AP	Pond DN	Pond AP	Pond DN	Pond AP	Pond DN
1	pH	-	6.73±0.8	6.75±0.5	6.73±0.2	6.75±0.7	6.73±0.3	6.75±0.8	6.73±0.5	6.75±0.
2	Turbidity	NTU	12±1.2	13±0.9	12±1.5	13±2.5	12±1.5	13±1.2	12±2.1	13±1.8
3	Total Hardness as $CaCO_3$	ppm	2000±30	1990±40	2000±50	1990±100	2000±70	1990±30	2000±110	1990±55
4	Total Alkalinity as $CaCO_3$	ppm	100±10	90±5.5	100±7.9	90±12	100±15	90±10	100±8.5	90±10
5	Nitrate as NO_3	ppm	3.92±0.5	3.89±0.1	3.92±0.5	3.89±0.4	3.92±0.5	3.89±0.7	3.92±0.8	3.89±0.8
6	Total suspended solids	mg/l	28±5	30±4	28±5	30±5	28±3	30±4	28±5	30±2
7	Chemical oxygen demand as O_2	ppm	46±0.5	58±0.6	45±0.9	58±0.8	46±1	58±1.2	46±0.8	58±1
8	Sulphide as S_2-	ppm	BDL (DL0.04)	BDL (DL0.04)	BDL (DL0.04)	BDL (DL0.04)	BDL (DL0.04)	BDL (DL0.04)	BDL (DL0.04)	BDL (DL0.04)
9	Dissolved oxygen	ppm	6±0.8	6±0.8	6±0.3	6±0.8	6±0.6	6±0.9	6±0.3	6±0.5
10	Ammonia as NH_3	ppm	BDL (DL0.04)	BDL (DL0.04)	BDL (DL0.04)	BDL (DL0.04)	BDL (DL0.04)	BDL (DL0.04)	BDL (DL0.04)	BDL (DL0.04)
11	Salinity	ppt	4.01±0.6	4±0.3	4.01±0.6	4±0.3	4.01±0.8	4±0.4	4.01±0.3	4±0.4
12	Total Nitrogen as N	ppm	18.66±0.3	18.65±0.6	18.66±0.7	18.65±0.3	18.66±0.5	18.65±0.1	18.66±0.5	18.65±0.3
13	BOD as O_2	ppm	7±0.2	11±0.6	7±0.5	11±0.4	7±0.8	11±0.5	7±0.5	11±0.4
14	Redox potential	mV	74±0.5	76±0.4	74±0.7	76±0.2	74±0.8	76±0.4	74±0.8	76±0.4

Table 4. Physico-chemical parameters of sediment in AP and DN ponds

S. no.	Soil parameters	Units	0-before stocking		30 days		60 days		Harvest	
			Pond AP	Pond DN	Pond AP	Pond DN	Pond AP	Pond DN	Pond AP	Pond DN
1	pH		6.8±0.5	6.8±0.4	6.8±0.8	6.7±0.7	6.8±0.5	6.7±0.8	6.8±0.5	7±0.5
2	Bulk density	g/cc	0.37±0.5	0.32±0.5	0.36±0.6	0.31±0.7	0.32±0.5	0.25±0.4	0.32±0.5	0.25±0.8
3	Water holding capacity	%	140±5	138±6	138±5	129±5	135±9	102±4	131±7	98±9
4	Lime requirement*	kg/hectare	500±15	500±20	500±25	500±15	500±25	500±30	500±15	500±23
5	Redox potential	mV	138±10	103±18	144±21	107±16	140±15	98±15	130±18	78±15
6	Sulphide		Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
7	Av. Nitrogen	mg/100g	16.3±3	16.2±3	17.2±5	19.4±4	16.1±3	18.6±2	14±3	16.9±3
8	Av. Phosphorous	mg/100g	1.6±0.6	2.5±0.4	2.2±0.3	2.1±0.4	2±0.6	1.9±0.3	1.6±0.5	1.4±0.4

**Lime requirement based on near neutral ph values observed in the samples*

Table 5. Correlation analysis of *Vibrio* spp., load with water and soil parameters using ANOVA

Variable	Source	SS	Df	Mean sq.	F
Vibrio spp. (Pond AP)	Water	28256	3	2173571	2.17
	Sediment	8900	3	1271.51	46.57
	Water×Sediment	28334.853	6	764.0322	0.588
Vibrio spp. (Pond DN)	Water	36734	2	245155	3
	Sediment	9100	2	1891	53.56
	Water×Sediment	46782	4	657.56	4

Sonia et al. [14] observed that the threshold level for *V. parahaemolyticus* was lower than 104 CFU/ml at harvesting period. Pattukumar et al. [15] showed *V. parahaemolyticus* load at harvesting time to be 1×10^2 and 1×10^3 water and sediment samples, respectively, from shrimp ponds in Cuddalore district, Tamil Nadu. They also added that congenial pond environment for shrimp culture could be retrieved using probiotics to minimize the *V. parahaemolyticus* load. Our results revealed that water samples contained relatively lower bacterial load than that of sediments. This depicts that the water samples reported much retarded increase than sediments. This finding is concordant with recently published report from Angela et al. [10].

4. CONCLUSION

Vibrio spp. distribution in water and sediment samples was evaluated from two shrimp ponds (Anuppampattu, AP and Devadanam, DN) of two different villages, Pullikulam and Kumarasirulapakkam, from Thiruvallur district. The results showed that the total *Vibrio* was one tenth of total bacterial load. The *Vibrio* spp. was identified as *V. paraheamolyticus* using 16s rRNA sequences. Their content varies with the increase of culturing time but was kept at threshold level by providing probiotics and maintaining the quality parameters at optimal level. Our findings thus conclude that the importance of quality parameters in subsiding the shrimp's opportunistic pathogen, *V. paraheamolyticus* for maximum productivity and better nutrient values. Further research is required on the effect of deteriorated water parameters on shrimp immunity and disease outbreaks [16].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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