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# Antibiotic resistance of culturable heterotrophic bacteria isolated from shrimp (*Penaeus vannamei*) aquaculture ponds

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ABSTRACT

Shrimp aquaculture is one of the fastest growing food-producing avenues, where antibiotics usage has become an issue of great concern due to the development of antimicrobial resistance in bacteria. A total of 2304 bacterial isolates from 192 samples (sediment, water, shrimp, and source water) from Andhra Pradesh, India were screened. Antibiotic resistance of bacterial isolates was highest for oxytetracycline (23.4%) followed by erythromycin (12.7%), co-trimoxazole (10%) ciprofloxacin (9.6%), and chloramphenicol (6%), of which 11.9% isolates were multi-drug resistant. Bacterial isolates from shrimp (26.7%), water (23.9%), and sediment (19.6%) samples exhibited more resistance ($p \leq 0.05$) towards oxytetracycline. Higher antibacterial resistance was observed from samples of southern Andhra Pradesh (locations L6 and L7). Gram negative bacteria were more prevalent (64%) and showed significantly ($p \leq 0.01$) higher resistance. This study indicated the wider distribution of antibiotic-resistant bacteria in shrimp aquaculture ponds with potential risk to humans and the environment.

## 1. Introduction

Aquaculture is one of the rapidly growing food sectors in the world which contributes substantially to food, nutrition, and livelihood security. The global fish production has grown to 179 million tonnes (valued at USD 401 billion), out of which aquaculture contributed 46% in terms of quantity (82 million tonnes) and 62% in terms of value (250 billion USD) (FAO, 2020). Marine and coastal aquaculture together produced 30.8 million tonnes of aquatic animals that are worth 106.5 billion USD. Presently, *Penaeus vannamei* (white leg shrimp) is the dominant species of cultured shrimps that can be easily farmed in brackish water, freshwater, and arid saline-alkaline waters. Production of *P. vannamei* is 4.96 million tonnes that contributes to 52.9% of the total crustacean production (FAO, 2020). In India, the culture of *P. vannamei* has expanded to 100,206 ha by 2017–18 which accounts for 622,000 t of production (Salunke et al., 2020; MPEDA, 2018). Among different shrimp producing states of India, Andhra Pradesh is the leading state in both *P. vannamei* production (456,000 t) and in the cultured area (62,000 ha) (MPEDA, 2018).

Antimicrobials are used in aquaculture worldwide, especially in intensive culture systems for growth promotion (Thornber et al., 2020) and in control of diseases (Romero et al., 2012; Rakesh et al., 2018). The incidence of diseases in *P. vannamei* culture in different regions drives the farmers to use antibiotics, bactericidal and chemical drugs (Mishra et al., 2017). In shrimp aquaculture, antibiotics such as ciprofloxacin, chloramphenicol, erythromycin, co-trimoxazole and tetracycline are commonly used (Costa et al., 2015). Excessive application of antibiotics in food production sectors has caused health problems and there is great concern on the emergence and dissemination of microbial resistance (WHO, 2016). In the recent years, despite strict regulations on antibiotics use in shrimp aquaculture, several consignments of shrimp exports were rejected due to the presence of pharmacologically prohibited substances including antibiotics (Panda and Ravishankar, 2018). Several studies reported antibiotic resistance of bacteria isolated from *P. monodon* farming (Kitiyodom et al., 2010; Hossain et al., 2012; Hua and Apun, 2013; Abraham, 2016; Kathleen et al., 2016; Narayanan et al., 2020). Similarly, several authors have also reported antimicrobial resistance (AMR) in bacteria from *P. vannamei* culture systems, but their

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studies centred on specific pathogens that were of public health significance (Banerjee et al., 2012; Stalin and Srinivasan, 2016; Teng et al., 2017; Dhayanath et al., 2019; Jiang et al., 2020). Cultured aquatic environments may be considered as a pool of antibiotic-resistant bacteria and emerging pollutant for antibiotic resistance (Marti et al., 2014) and therefore the specific-pathogen focussed approach does not provide the complete picture on the prevalence of drug-resistant bacteria in the aquatic animal food production system.

In tropical regions, culturable heterotrophic bacterial populations prevail in higher densities than the autotrophic bacterial populations, as the conditions are congenial for the rapid proliferation of heterotrophs. Moreover, the isolation and characterization of heterotrophs in terms of antibiotic resistance is easier in comparison to the specific pathogen strategy. The heterotrophic bacteria have been used as indicator organisms for determining drug resistance in water quality surveillance programs (Huys et al., 2007). The data on antibiotic resistance of these heterotrophic bacteria will help in elucidating their role in the acquisition of resistance and its dissemination as there is large scale horizontal gene transfer by mobile genetic elements among the heterotrophic bacteria in aquatic environs (Von Wintersdorff et al., 2016). However, there is a paucity of information available on the prevalence of antimicrobial resistance in culturable heterotrophic bacteria from *P. vannamei* and its culture environment. Hence, the present study is undertaken to assess the heterotrophic bacteria from shrimp (*P. vannamei*) culture ponds in different geographical locations of Andhra Pradesh, India and to study their resistance against five commonly used antibiotics viz., ciprofloxacin, chloramphenicol, erythromycin, co-trimoxazole and oxytetracycline.

## 2. Materials and methods

### 2.1. Study locations

A total of 180 samples comprising of pond sediment (n-60), farm water (n-60) and farmed shrimp (n-60) from sixty shrimp ponds in 12 different geographical locations of Andhra Pradesh, India was investigated over a period of 24 months (January 2017 to December 2018). The shrimp ponds were 4000 to 6500 $m^2$ in size with water depth ranging from 1.5 to 2.5 m and stocked with post-larvae at a density of 10 to 40 shrimp per $m^2$. The shrimps were fed with commercial pellet feeds and provided with continuous aeration (Supporting Tables S1A–S1F). The shrimp ponds were located in four districts namely Srikakulam (L1, L10), East Godavari (L2, L3, L4, L5, L11 and L12), West Godavari (L8, L9) and Sri Potti Sriramulu Nellore (L6, L7) districts of Andhra Pradesh (Fig. 1). In each of the pond location, three samples of sediment (S), water (W), and shrimp (Sh) were drawn and designated as L1ASh, L1AW, L1AS; L1BSh, L1BW, L1BS; L1CSh, L1CW, L1CS; L1DSh, L1DW, L1DS; L1ESh, L1EW, L1ES (A, B, C, D, E are five shrimp ponds) for the location L1 and a similar pattern was followed for all other locations (L2−L12), respectively. In addition, source water (SW) samples ((n-12) (reservoir pond) were also collected from respective pond sites and designated as L1SW to L12SW for location L1 to L12 (one water sample for each location). The management of ponds from locations L1 to L11 was carried out by aqua farmers in respective locations and the minimum distance between any two sampling ponds was 2 to 6 km, wherein the information available on the use of antibiotics was uncertain during the rearing of shrimps. The ponds at location L12 (control ponds) were operated by a federal agency (ICAR-Central Institute of Fisheries Education, Kakinada), in which antibiotics were not employed during the

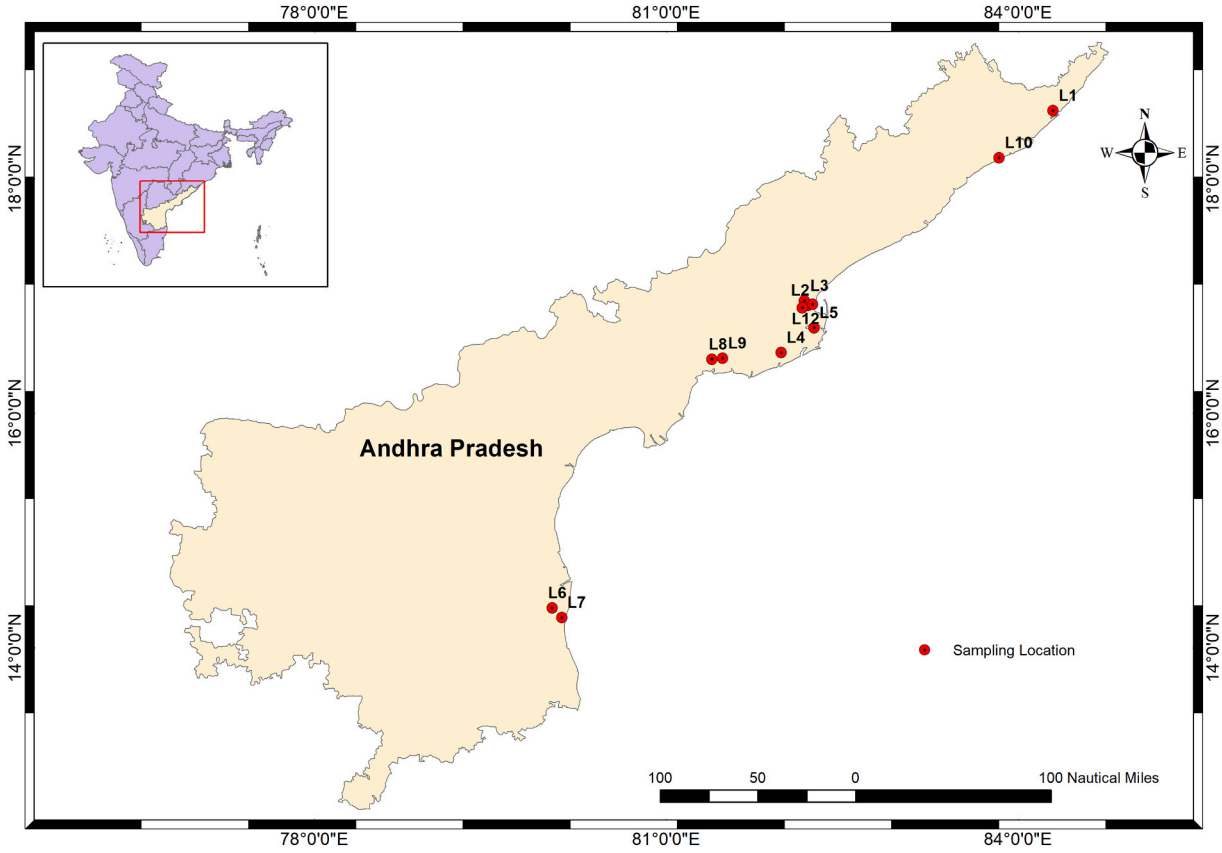


**Fig. 1.** Map showing different sample collection locations of Andhra Pradesh, India where the shrimp ponds were located (Inset: Map of India highlighting Andhra Pradesh). L1- Location 1 (18.58′N; 84.30′E); L2- Location 2 (16.92′N; 82.21′E); L3- Location 3 (16.96′N; 82.18′E); L4- Location 4 (16.52′N; 81.98′E); L5- Location 5 (16.73′N; 82.21′E); L6 (14.34′N; 80.03′E); L7- Location 7 (14.26′N; 80.11′E); L8- Location 8 (16.46′N; 81.39′E); L9- Location 9 (16.47′N; 81.49′E); L10- Location 10 (18.18′N; 83.84′E); L11- Location 11 (16.90′N; 82.16′E); L12- Location 12 (16.93′N; 82.25′E).

rearing of animals.

### 2.2. Sample collection

The samples from shrimp culture ponds were collected for each location separately as per the procedure described by Tendencia and De la Peña (2001). From each sampled pond, farm water was collected from three different places (near the water inlet from where the water enters the pond, from the middle of the pond, and near the outlet of pond from where the water exits) separately at a depth of 50 cm by using a 500 mL sterile water collection bottle and all three portions were pooled to represent a single composite water sample. Sediment samples (approx. 500 g) were collected from the surface layer (1–2 cm depth) of pond bottom at the same locations from where the farm water samples were collected, in a sterile sample collection bag and later pooled to a single sediment sample. Active sampling was performed, wherein healthy shrimps (8–10 animals) (appx. 4–8 g weight) which accumulated in the feed check tray were collected into a sterile sampling bag. The sampling was carried out from the standing crop of the respective locations, separately, at random, as the time schedule for crop season varied from location to location. Each sample was assigned with a unique code, after collection in sterile condition and transported to the laboratory for immediate analyses. Water quality parameters such as temperature, pH, and salinity were measured from all pond collection sites employing a multi-parameter detection probe (Hanna Instruments, USA). Other parameters namely dissolved oxygen (D.O.), alkalinity, total hardness, ammonia, nitrite, and hydrogen sulphide were obtained from the field logbook.

### 2.3. Isolation and enumeration of total culturable heterotrophic bacteria

Isolation and enumeration of culturable heterotrophic bacteria from samples were carried out by surface plate technique as per ISO 4833-2: 2013 with minor modifications (marine agar was employed instead of plate count agar). One gram or 1 mL of sample (composite sediment, water samples and pooled shrimp meat without head) was homogenized in 9 mL of sterile phosphate buffer saline (PBS, pH 7.2), serially diluted, spread plated on marine agar (BD Difco, USA) in duplicate and incubated for 24–48 h at 30 °C in the incubator (Thermoscientific, USA). For data analyses, counts from five ponds in a single location were taken and the means were compared. As described in ISO 4833-2: 2013 representative bacterial colonies (30 to 40 distinct morphological colonies) were randomly selected from the plates for each sample, purified and maintained on marine agar slant for further studies on antibiotic resistance. Additionally, all the purified isolates were preserved in 30% glycerol and stored at -80 °C.

### 2.4. Antibiotic sensitivity studies

Antibiotic susceptibility test of bacteria was carried out by disc diffusion method (CLSI, 2020) against five classes of antibiotics namely tetracyclines, macrolides, folate pathway inhibitors, quinolones, and phenicols. *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) were employed as quality control strains. All bacterial isolates were inoculated into Mueller-Hinton (MH) broth and incubated overnight (16–18 h) at 35 °C. The grown culture was centrifuged at 5000 rpm for 5 min at 25 °C and the supernatant was discarded. The bacterial pellet was dissolved in PBS (pH 7.2) and the optical density ($OD_{600}$) was adjusted to 0.5. The bacterial suspension was spread plated onto MH agar using sterile cotton swabs and kept for 5 min for drying before placing the antibiotic discs. The plates were then incubated at 35 ± 1 °C for 18–24 h. After incubation, the zone of inhibition was measured and the resistance pattern was interpreted as per CLSI (2020). The antibiotics discs (Himedia, India) used in this study and their concentrations were: ciprofloxacin (5 μg); chloramphenicol (30 μg); cotrimoxazole (10 μg); erythromycin (15 μg) and oxytetracycline (30 μg).

### 2.5. Morphotintorial categorization of bacterial isolates by Gram staining

Gram staining of the bacterial isolates from sediment, water, and shrimp was performed as described by Chauhan and Jindal (2020) and morphotintorial categorization was carried out.

### 2.6. Data analysis

Statistical analysis of the data was carried out by using SPSS (SPSS 20, USA) software. Two-way Analysis of Variance (ANOVA, Duncan's) was applied to test the significant differences in mean culturable heterotrophic plate counts (HPC) and antibiotic resistance of bacteria isolated from different samples and locations. Pearson correlation coefficient (r) was used to analyse the correlation of antibiotic resistance of bacteria isolated from sediment, water, and shrimp. The paired *t*-test was used to test the significant differences in antibiotic resistance patterns of bacterial groups based on Gram staining.

## 3. Results

### 3.1. Water quality parameters

Water quality parameters of shrimp ponds during the present study are presented in supporting Tables S1A to S1F. Variations in the water quality parameters of the shrimp ponds were minimal. Water temperature, dissolved oxygen, and pH were in the range of 29–32 °C, 5–6 mg $L^{-1}$ and 7.6–8.2, respectively at all the locations. Alkalinity and total hardness were observed in the range of 100–250 mg $L^{-1}$ and 1250–3600 mg $L^{-1}$, respectively. However, a wide variation was recorded for water salinity with a minimum of 4 psu in location L3 and a maximum of 32 psu in location L7. Other water quality parameters such as ammonia, nitrite, and hydrogen sulphide were not detected in any of the locations.

### 3.2. Enumeration of total culturable heterotrophic bacteria

The HPC of pond sediment, farm water and farmed shrimp samples collected from shrimp ponds is shown in Fig. 2. Significant difference ($p \leq 0.05$) was observed in the HPC among the samples in different locations. The mean HPC from the sediment samples was recorded in the range of 6.3 to 6.4 $Log_{10}$ CFU $g^{-1}$ with highest count from the ponds in location L5 (Fig. 2a). In case of water samples, the mean HPC was observed in the range of 4.2 to 4.4 $Log_{10}$ CFU $mL^{-1}$ with the highest count from the ponds in location L11 (Fig. 2b). Similarly, in the case of shrimp samples, the mean HPC was observed in the range of 5.3 to 5.4 $Log_{10}$ CFU $g^{-1}$ with the highest count from ponds in location L11 (Fig. 2c).

### 3.3. Resistance pattern of bacterial isolates to different antibiotics

A total of 2304 bacterial isolates from shrimp pond samples (sediment, water, shrimp, and source water) were evaluated for resistance against five different antibiotics. Bacterial isolates exhibited the highest resistance towards oxytetracycline (23.4%) followed by erythromycin (12.7%) (Fig. 3). Among the bacterial isolates from source water samples, the highest resistance was observed for erythromycin (5.6%) followed by oxytetracycline (3.5%). Among all the antibiotic resistant bacteria from shrimp ponds, 11.9% of isolates were multi-drug resistant (MDR). Similarly, 1.4% of the resistant bacterial isolates from source water (SW) samples showed MDR.

### 3.4. Antibiotic resistance of bacteria isolated from farm sediment, pond water and farmed shrimp samples

The data pertaining to antibiotic resistance (percentage) of bacteria isolated from sediment, water, and shrimp samples is depicted in Fig. 4. The bacteria isolated from shrimp samples (26.7%) exhibited higher

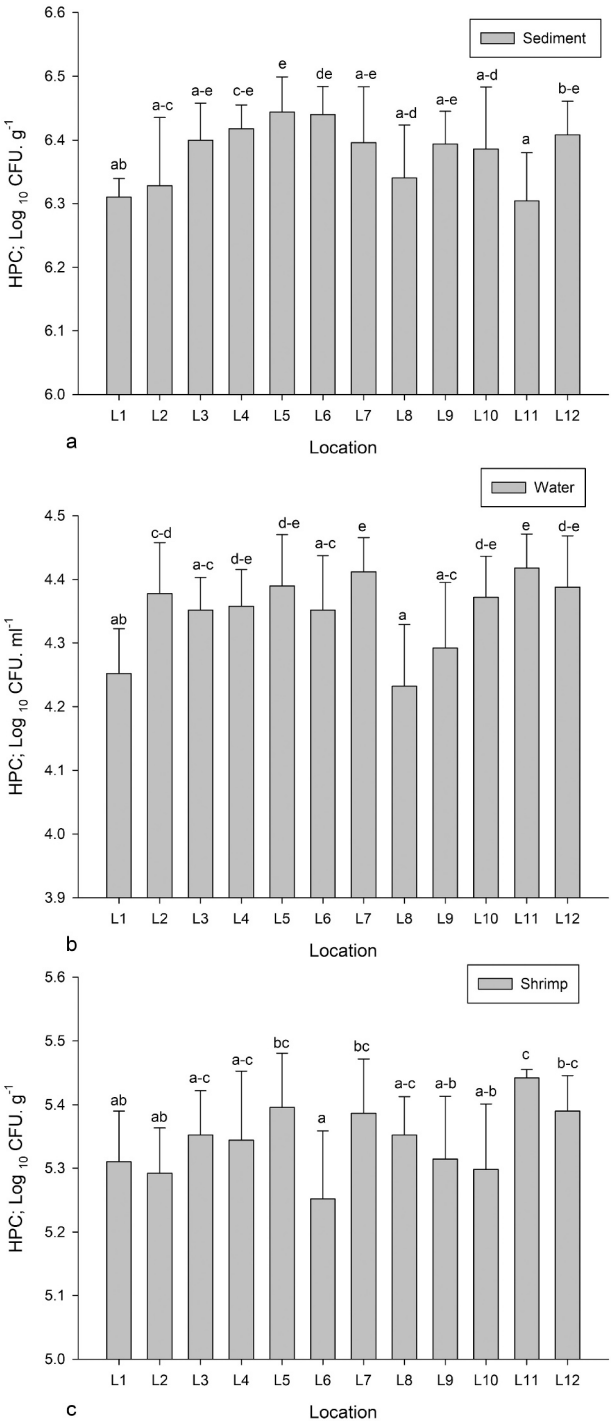


**Fig. 2.** a. Mean HPC ($Log_{10}$ CFU $g^{-1}$) of sediment samples collected from shrimp ponds of different locations (L1–L12). Values (mean $Log_{10}$ CFU $g^{-1}$ ± SD, n = 5) containing different superscripts denotes significant difference among the locations ($p \leq 0.05$).
b. Mean HPC ($Log_{10}$ CFU $mL^{-1}$) of water samples collected from shrimp ponds of different locations (L1–L12). Values (mean $Log_{10}$ CFU $mL^{-1}$ ± SD, n = 5) containing different superscripts denotes significant difference among the locations ($p \leq 0.05$).
c. Mean HPC ($Log_{10}$ CFU $g^{-1)}$ of shrimp samples collected from shrimp ponds of different locations (L1–L12). Values (mean $Log_{10}$ CFU $g^{-1}$ ± SD, n = 5) containing different superscripts denotes significant difference among the locations ($p \leq 0.05$).

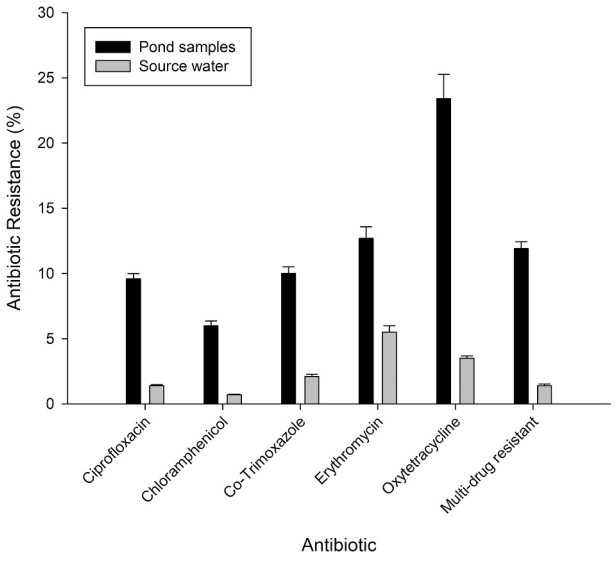


**Fig. 3.** Antibiotic resistance (Mean ± SD) of bacteria isolated from shrimp pond samples and source water samples to different antibiotics.

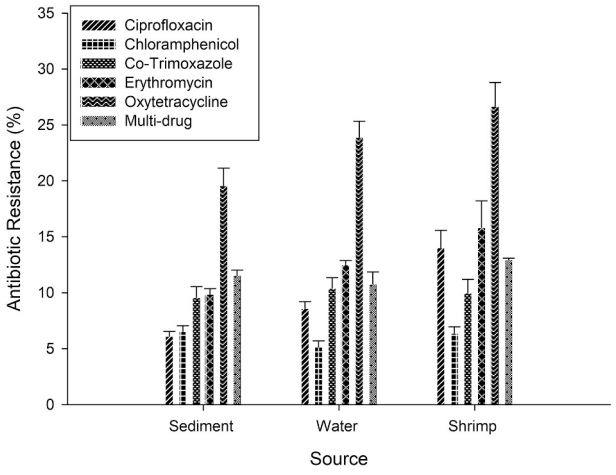


**Fig. 4.** Antibiotic resistance (Mean ± SD) of bacteria isolated from farm sediment, pond water and farmed shrimp samples.

resistance towards oxytetracycline compared to the water (23.9%) and sediment (19.6%) samples and were significantly different ($p \leq 0.01$). Pearson correlation coefficient analysis showed significantly positive correlation in the antibiotic resistance of bacterial isolates from the pond sediment and farm water ($r = 0.979$, $p \leq 0.05$); pond sediment and farmed shrimp ($r = 0.895$, $p \leq 0.05$) and pond water and farmed shrimp ($r = 0.962$, $p \leq 0.05$). Among the three categories of samples, multi-drug resistance was relatively higher in farmed shrimp (12.9%) compared to pond sediment (11.5%) and farm water (10.7%).

### 3.5. Location-wise variation in antibiotic resistance

The antibiotic resistance (percentage) of bacterial isolates from sediment, water, and shrimp samples at different locations (L1−L12) of shrimp ponds is depicted in Fig. 5. Among the sediment samples from all locations, antibiotic resistance was observed highest at location L6 (ciprofloxacin-18.3%; chloramphenicol-15%; co-trimoxazole-31.7%; erythromycin-25% and oxytetracycline-60%) (Fig. 5a). Similarly, for water samples collected from all locations, maximum resistance was noted at the location L6 to all antibiotics (ciprofloxacin-25%;

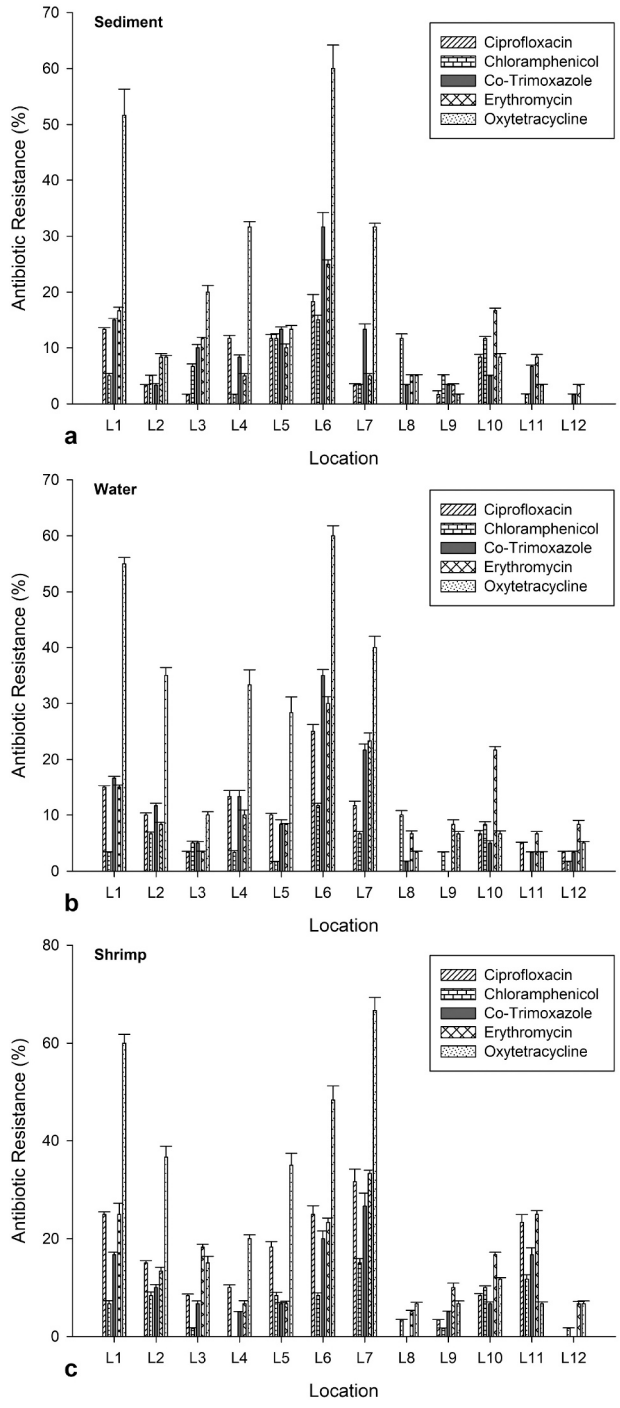


**Fig. 5.** a. Location-wise variations in antibiotic resistance (Mean ± SD) of bacteria isolated from farm sediment samples.
b. Location-wise variations in antibiotic resistance (Mean ± SD) of bacteria isolated from pond water samples.
c. Location-wise variations in antibiotic resistance (Mean ± SD) of bacteria isolated from farmed shrimp samples.

chloramphenicol-11.7%; co-trimoxazole-35%; erythromycin-30% and oxytetracycline-60%) (Fig. 5b). However, bacterial isolates from shrimp samples at location L7 showed peak resistance to all antibiotics (ciprofloxacin-31.7%; chloramphenicol-15%; co-trimoxazole-26.7%; erythromycin-33% and oxytetracycline-66.7%) (Fig. 5c). In location L12 the bacterial isolates from all the samples showed the lowest resistance (%) to the tested antibiotics. There exists a significant difference ($p \leq 0.01$) among the locations to antibiotic resistance of bacteria isolated from farm sediment, pond water, and farmed shrimp samples.

### 3.6. Pond-wise differences in antibiotic resistance

The differences in pond-wise antibiotic resistance (percentage) of bacteria sourced from sediment, water, and shrimp samples were analysed and shown in Table 1 and Supporting Table S2. The utmost resistance percentage (mean ± SD) was shown by bacteria isolated from sediment (60 ± 21.5), water (60 ± 13.6), and shrimp (66 ± 18) towards oxytetracycline (Table 1). The highest resistance to ciprofloxacin was observed from the shrimp sample of pond L7 E (58.3%), whereas, highest antibiotic resistance for chloramphenicol was seen in the bacterial isolates from sediment sample of pond L5 E (41.67%). In case of co-trimoxazole, the percent resistance was highest for bacterial strains isolated from water samples in the pond L6 D (75.0%). Similarly, the antibiotic resistance for erythromycin (66.67%) and oxytetracycline (100.0%) was found to be high in bacterial isolates from shrimp samples in pond L7 D (Supporting Table S2).

### 3.7. Antibiotic resistance among morphotintorial groups

All the bacterial isolates from sediment, water, and shrimp samples were categorized by Gram staining into two morphotintorial groups: Gram positive (GP) and Gram negative (GN) (Table 2). The majority of bacteria isolated from shrimp farms belonged to GN (64.2%). The antibiotic resistance in GP ranged from 5 to 82% with a maximum at location L6. In the case of GN rods, the antibiotic resistance varied from 6.1 to 70.6% and was highest in location L6. Similarly, GN antibiotic resistance ranged from 15.5 to 82.8% with extremity in locations L6 and L1. A Significant difference was observed in the antibiotic resistance of GP and GN (paired *t*-test, $p \leq 0.01$).

## 4. Discussion

*P. vannamei* shrimp is cultured globally and contributes significantly to the world economy (FAO, 2020). A remarkable growth in production of *P. vannamei* has been witnessed in India since 2009 and among all the states in India that farm *P. vannamei*, the state of Andhra Pradesh leads in production and area under culture (MPEDA, 2018). All the water quality parameters recorded in this study were optimal, indicating that there was no stress during the rearing of shrimps. Saraswathy et al. (2019) have indicated that the significant deviations in water quality parameters may act as stress to the aquatic animals under intensive culture systems. However, few deviations were seen in water alkalinity and hardness. Pond waters with higher alkalinity have a greater concentration of metal ions which are essential for microbial growth. Similarly, the pond water with low alkalinity inhibits the microbial decomposition of organic matter at the pond bottom, thereby changing the microbial composition in the pond sediments. Similarly, both water alkalinity and hardness had a profound influence on the microbial populations involved in the nitrogen and sulphur cycles (Boyd et al., 2016). Beneficial bacterial species such as *Bacillus* are involved in maintaining alkalinity and reducing water hardness in aquaculture ponds. Sporulation of the bacterial species has also been affected due to the high hardness of pond waters (Hlordzi et al., 2020).

Water quality considerably influences the microbial load of an aquaculture pond and the diversity of bacteria varies based on different parameters (Qin et al., 2016). In this study, the culturable HPC of farm sediment, pond water, and farmed shrimp were in the range of 6.18 to 6.52 $Log_{10}$ CFU $g^{-1}$, 4.14 to 6.5 $Log_{10}$ CFU $mL^{-1}$ and 5.11 to 5.51 $Log_{10}$ CFU $g^{-1}$ respectively and are on par with the total bacterial counts reported by several authors (Raja et al., 2017; Joseph et al., 2017; Tawade et al., 2019). A significant difference ($p \leq 0.5$) was noticed between the mean counts at different locations. Sediment samples recorded high culturable heterotrophic bacteria compared to rearing water and shrimp. Shrimp aquaculture ponds with high stocking densities employ

**Table 1**
Pond wise differences in antibiotic resistance (Mean ± SD) of bacteria isolated from different samples.

| | Antibiotic resistance (%) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sediment (S) | | | | | Water (W) | | | | | Shrimp (Sh) | | | | |
| Pond | CIP | C | COT | ERM | OTC | CIP | C | COT | ERM | OTC | CIP | C | COT | ERM | OTC |
| A, B, C, D, E (L1) | 13.3 ± 9.5 | 5.0± 11.1 | 15.0 ± 20.7 | 16.6 ± 14.4 | 51.6 ± 22.3 | 15.0 ± 6.9 | 3.3 ± 7.4 | 16.6 ± 5.8 | 15.0 ± 12.3 | 55.0 ± 17.2 | 25.0 ± 19.5 | 6.6 ± 6.9 | 16.6 ± 15.5 | 25.0 ± 11.7 | 60.6 ± 18.6 |
| A, B, C, D, E (L2) | 3.3 ± 4.5 | 5.0 ± 7.4 | 3.3 ± 7.4 | 8.3 ± 8.3 | 8.3 ± 8.3 | 10.0 ± 10.8 | 6.6 ± 9.1 | 11.6 ± 17.2 | 8.3 ± 8.3 | 35.0 ± 33.5 | 15.0 ± 17.0 | 8.3 ± 8.3 | 10.0 ± 13.6 | 13.3 ± 12.6 | 36.6 ± 32.6 |
| A, B, C, D, E (L3) | 1.6 ± 3.7 | 6.6 ± 10.8 | 10.0 ± 10.8 | 11.6 ± 9.5 | 20.0 ± 16.2 | 3.3 ± 7.4 | 5.0 ± 7.4 | 5.0 ± 7.4 | 3.3 ± 4.5 | 10.0 ± 13.6 | 8.3 ± 0.0 | 1.6 ± 3.7 | 6.6 ± 10.8 | 18.3 ± 9.1 | 15.0 ± 6.9 |
| A, B, C, D, E (L4) | 11.6 ± 4.5 | 1.6 ± 3.7 | 8.3 ± 8.3 | 5.0 ± 4.5 | 31.6 ± 21.5 | 13.3 ± 9.5 | 3.3 ± 4.5 | 13.3 ± 11.1 | 10.0 ± 3.7 | 33.3 ± 13.1 | 10.0 ± 6.9 | 0.0 ± 0.0 | 5.0 ± 7.4 | 6.6 ± 6.9 | 20.0 ± 12.6 |
| A, B, C, D, E (L5) | 11.6 ± 17.2 | 11.6 ± 18.2 | 13.3 ± 13.9 | 10.0 ± 10.8 | 13.3 ± 20.9 | 10.0 ± 9.1 | 1.6 ± 3.7 | 8.3 ± 11.7 | 8.3 ± 8.3 | 28.3 ± 26.0 | 18.3 ± 16.03 | 8.3 ± 5.8 | 6.6 ± 10.8 | 6.6 ± 6.9 | 35.0 ± 31.4 |
| A, B, C, D, E (L6) | 18.3 ± 10.8 | 15.0 ± 6.9 | 31.6 ± 9.1 | 25.0 ± 10.2 | 60.0 ± 21.5 | 25.0 ± 11.7 | 10.0 ± 9.1 | 35.0 ± 23.8 | 30.0 ± 15.1 | 60.0 ± 13.6 | 25.0 ± 11.7 | 8.3 ± 5.8 | 26.6 ± 19.0 | 23.3 ± 6.9 | 66.0 ± 18.0 |
| A, B, C, D, E (L7) | 3.3 ± 4.5 | 3.3 ± 4.5 | 13.3 ± 9.5 | 5.0 ± 7.4 | 31.6 ± 23.1 | 11.6 ± 7.4 | 6.6 ± 3.7 | 21.6 ± 16.2 | 23.3 ± 10.8 | 40.0 ± 34.0 | 31.6 ± 29.1 | 15.0 ± 3.7 | 20.0 ± 13.9 | 33.3 ± 21.2 | 48.3 ± 39.7 |
| A, B, C, D, E (L8) | 0.0 ± 0.0 | 11.6 ± 4.5 | 3.3 ± 4.5 | 5.0 ± 4.5 | 5.0 ± 7.4 | 0.0 ± 0.0 | 11.6 ± 7.4 | 1.6 ± 3.7 | 6.6 ± 10.8 | 3.3 ± 4.5 | 0.0 ± 0.0 | 3.3 ± 4.5 | 0.0 ± 0.0 | 5.0 ± 4.5 | 6.6 ± 14.9 |
| A, B, C, D, E (L9) | 1.6 ± 3.7 | 5.0 ± 4.5 | 3.3 ± 4.5 | 3.3 ± 7.4 | 1.6 ± 3.7 | 0.0 ± 0.0 | 3.3 ± 7.4 | 0.0 ± 0.0 | 8.3 ± 10.2 | 6.6 ± 6.9 | 3.3 ± 7.4 | 1.6 ± 3.7 | 5.0 ± 11.1 | 10.0 ± 9.1 | 6.6 ± 14.9 |
| A, B, C, D, E (L10) | 8.3 ± 5.8 | 11.6 ± 7.4 | 5.0 ± 4.5 | 16.6 ± 14.4 | 8.3 ± 5.8 | 6.6 ± 3.7 | 8.3 ± 5.8 | 5.0 ± 4.5 | 21.6 ± 4.5 | 6.6 ± 3.7 | 8.3 ± 0.0 | 10.0 ± 9.1 | 6.6 ± 3.7 | 16.7 ± 10.2 | 11.6 ± 7.4 |
| A, B, C, D, E (L11) | 0.0 ± 0.0 | 1.6 ± 3.7 | 6.6 ± 10.8 | 8.3 ± 0.0 | 3.3 ± 7.4 | 5.0 ± 4.5 | 0.0 ± 0.0 | 3.3 ± 7.4 | 6.6 ± 6.9 | 3.3 ± 4.5 | 23.3 ± 6.9 | 11.6 ± 9.5 | 16.6 ± 5.8 | 25.0 ± 13.1 | 6.6 ± 6.9 |
| A, B, C, D, E (L12) | 0.0 ± 0.0 | 0.0 ± 0.0 | 1.6 ± 3.7 | 3.3 ± 4.5 | 0.0 ± 0.0 | 3.3 ± 4.5 | 1.6 ± 3.7 | 3.3 ± 4.5 | 8.3 ± 11.7 | 5.0 ± 7.4 | 0.0 ± 0.0 | 1.6 ± 3.7 | 0.0 ± 0.0 | 6.6 ± 10.8 | 6.6 ± 10.8 |

CIP- ciprofloxacin, C-chloramphenicol, COT-co-trimoxazole, ERM-erythromycin and OTC-oxytetracycline; (A, B, C, D, E) five ponds from each location; L-Location.

**Table 2**
Morphotintorial categorization of bacterial isolates from different locations and their antibiotic resistance.

| Location | GP (n-825) | GN (n-1479) | Antibiotic resistant GP (%) | Antibiotic resistant GN (%) |
|---|---|---|---|---|
| L1 | 63 | 117 | 76.2 | 68.3 |
| L2 | 65 | 115 | 43.1 | 67.8 |
| L3 | 63 | 117 | 28.6 | 58.9 |
| L4 | 64 | 116 | 60.9 | 68.9 |
| L5 | 64 | 116 | 51.6 | 58.6 |
| L6 | 64 | 116 | 70.6 | 82.8 |
| L7 | 64 | 116 | 57.8 | 63.7 |
| L8 | 64 | 116 | 18.8 | 67.2 |
| L9 | 65 | 114 | 16.9 | 59.6 |
| L10 | 64 | 117 | 32.8 | 52.1 |
| L11 | 65 | 115 | 20.0 | 64.3 |
| L12 | 66 | 114 | 18.2 | 6.1 |
| SW | 54 | 90 | 14.8 | 15.5 |
| Total (%) | 35.8 | 64.2 | | |

GP-Gram positive; GN-Gram negative. Total (%) indicates composition of each morphotintorial group to total bacterial isolates; L-Location; SW-source water.

more amount of pelleted feeds to maximize the production and the unused feed gets deposited at the pond bottom leads to proliferation of microbial population in the pond sediments (Joseph et al., 2017).

Several studies reported antimicrobial usage in shrimp aquaculture (Romero et al., 2012; Sundaramanickam et al., 2015; Song et al., 2016). The rampant usage of antimicrobials resulted in the prevalence of antimicrobial resistant bacteria as well as multiple antibiotic resistance in aquatic environments (Mudryk et al., 2010; Cabello et al., 2013). Similarly, in this study too, the occurrence of antibiotic resistant bacteria was observed in *P. vannamei* culture environment. Among the five antibiotics studied, antibiotic resistance of bacterial isolates was highest for oxytetracycline (23.38%). Likewise, higher tetracycline resistance of 57.1%, 36.2%, 13%, and 8.2% in bacterial isolates from shrimp and its culture environment have been reported by Gao et al. (2012); Liu et al. (2019); Zhang et al. (2011) and Kathleen et al. (2016), respectively. The reasons being tetracyclines are one of the most frequently employed antibiotics in aquaculture (Prasad and Ravishankar, 2018) and are inexpensive compared to other antibiotics used in aquaculture (Gao et al., 2012). Besides, AMR was also accounted for erythromycin (12.73%), co-trimoxazole (10%), ciprofloxacin (9.58%), and chloramphenicol (6.02%). Chloramphenicol, ciprofloxacin, co-trimoxazole, and erythromycin are widely used for treating diseases in humans and animals and the resistance for these antibiotics in aquaculture environments is quite alarming (Thuy et al., 2011).

The results from this study indicate that bacteria isolated from sediment, water, and shrimp samples collected from *P. vannamei* culture ponds show a significant level of resistance ($p \leq 0.05$) to oxytetracycline compared to other screened antibiotics and is likely due to its extensive use in shrimp farms (Prasad and Ravishankar, 2018). Several reports indicate the highest resistance towards oxytetracycline in bacteria from the marine environment (Buschmann et al., 2012; Shah et al., 2014). Among the sediment, water, and shrimp samples, the percent antibiotic resistance in shrimp samples was higher for the possible reason that

these antibiotics were administered through the feed. When shrimp consumes the feed, these substances get accumulated and result in the development of microbial resistance (Costa et al., 2015). Zhang et al. (2011) have also demonstrated the presence of antibiotic resistant bacteria in sediment and water samples collected from Donghai Island, China. Antibiotic resistant bacteria commonly found in coastal shrimp pond water and effluents have been reported by Wahid et al. (2015). The factors such as antibiotic residues in the culture environment and past usage of antibiotics in shrimp farms also determine the antibiotic resistance pattern in the environmental bacteria. Shrimp pond sediments harbour antibiotics for a longer duration than the pond water, which serves as a hub for further dissemination of antimicrobial resistance in the next cycle of the culture period (Zhang et al., 2011). The sediments in marine environments harbours more drug resistant bacteria similar to that of sediments of aquaculture ponds that is originated from the applied feed, feed supplements, and chemicals available in the organic manure (Smith, 2008). Buschmann et al. (2012) have also reported higher resistance of bacteria from the marine environment that was adjacent to aquaculture facilities.

The antibiotic resistance found in shrimp aquaculture systems is also on the higher side similar to marine environments as these are continuously subjected to different anthropogenic activities such as mixing of domestic sewage effluents, municipal wastewaters, hospital waste etc. (Lobova et al., 2002). Antibiotic resistance detected in aquaculture systems and in the marine environments have the same detrimental roles on human and aquatic animal health as there is wide transfer of resistant determinants from aquaculture-origin microbes to marine bacteria and finally to terrestrial pathogenic bacteria that cause infections in humans (Burridge et al., 2010). The potential implications of high antibiotic resistance in the heterotrophic bacterial populations includes the development of antibiotic-resistance in commensal and beneficial microorganisms which are non-pathogenic, acquiring of multi-drug resistance to different classes of antibiotics, resistance transfer to terrestrial opportunistic human pathogens, and reduced efficiency of antimicrobial treatment for control of infections caused by drug-resistant bacteria (Kathleen et al., 2016; Murugadas et al., 2019).

The results of this study also indicated that 11.85% of drug resistant bacteria from shrimp culture ponds were MDR to three or more classes of antibiotics (Magiorakos et al., 2012). Multiple antibiotic resistance (MAR) from aquaculture systems have also been reported in previous studies (Mudryk et al., 2010; Pham et al., 2018). Also, Zhang et al. (2011) have reported samples collected from shrimp ponds showing multiple resistance to 3–5 antibiotics studied. Occurrence of MDR in 30 *Vibrio* species isolated from different seafood items of Kerala, India has been reported (Manjusha and Sarita, 2013). Generally, antibiotics are commonly applied at sub-therapeutic levels for preventing or treating bacterial disease, increasing feed consumption, and faster growth of animals in shrimp ponds (Thornber et al., 2020). This study also established the prevalence of MAR in bacterial isolates and a positive correlation has been noticed between the soil, water, and shrimp samples. Hossain et al. (2012) have demonstrated a positive correlation between multiple antibiotic resistance and antibiotics used in aquaculture.

Antibiotic resistance of bacteria may vary with the geographical location, where selective pressure and resistance patterns change quickly from time to time (Satyanarayana, 2009). Among different shrimp farm locations studied, antibiotic resistance was maximum at locations L6 and L7 compared to all other locations. This is attributed to the differences in stocking densities followed in different locations and also the source of seed (post-larvae) contributes to the spread of resistance. In hatcheries, it is a common practice to use antibiotics as prophylactic agents (Zhang et al., 2011). Therefore, the same resistance pattern carried forward possibly to the culture ponds. In addition, the release of water during harvest and also removal of sediments during pond preparation increases the risk associated with high intensities of antibiotic pollution in the farming environments (Hoa et al., 2011; Hossain et al., 2017; Lai et al., 2018). The application of antibiotic supplemented commercial feeds initiates microbial resistance (Costa et al., 2015). In our study, in location L12 antibiotic resistance of bacterial isolates was relatively lower as these ponds were managed by federal agencies where the application of antibiotics for the growth of animals is prohibited. In all the locations, water used for rearing was also studied for drug resistant bacteria. The results have indicated that most bacterial isolates were sensitive to the antibiotics tested. For shrimp farming, water used for the rearing of animals is stored in reservoir tanks prior to the start of culture operations and also treated with lime which is the primary requirement as per the regulations of Coastal Aquaculture Authority, India (CAA, 2005). Occasionally, local water sources can also be highly polluted with antimicrobial substances and antibiotic resistant bacteria (Su et al., 2017). But, in this study, no such observation was made. Recently, there has been serious concern over the quality of the probiotics sold in the global markets with studies reporting microbial count differing from those listed on the label and many of them carrying antibiotic resistant bacteria to multiple antibiotics (Noor-Uddin et al., 2015; Uma and Rebecca, 2018).

Grams staining plays an important role in morphotintorial categorization of bacteria as the cell wall is differentiating character for the classification of bacteria as GN and GP. In addition, the available antibiotics target bacterial cells by different mechanisms that act on cell wall or cell membrane causing cell death. The morphological determinants also help in discovering novel antibiotic drug targets (van Teeseling et al., 2017). Among the different morphological groups, Gram negative bacteria (64%) were more prevalent in shrimp farms which were in accordance with the results demonstrated by previous reports (Zhang et al., 2011; Ahmed et al., 2015; Dhayanath et al., 2019) and showed higher resistance to the tested antibiotics. As shrimp aquaculture is carried out under controlled environment, any minor deviations in culture conditions induce stress in the animal and lower its immune response causing diseases. To mitigate this problem, aquaculturists employ antibiotics in shrimp culture ponds which had resulted in the development of resistance and more frequently in Gram negative bacteria compared to other groups of bacteria (Ahmed et al., 2015). The presence of antimicrobial resistance in these groups makes it an additional burden in controlling bacterial infections in aquaculture environments.

## 5. Conclusion

This study revealed the wide prevalence of antibiotic resistant culturable heterotrophic bacteria in shrimp culture ponds. Highest resistance was seen towards oxytetracycline and bacteria isolated from shrimp samples showed high resistance to the tested antibiotics compared to sediment and water samples. The differences in the incidence of antibiotic resistance of bacterial isolates in different locations may be attributed to several factors such as re-use of water from the previous crop sans treatment, stocking of seed (post-larvae) from different hatcheries, diverse culture practices, the release of effluent waters during harvest into surroundings and use of contaminated water source. Gram negative bacteria showed relatively higher drug resistance and the excess use of antibiotics in shrimp farming might disseminate multi-drug resistance to other heterotrophic bacterial populations through horizontal gene transfer. Therefore, the creation of proper awareness on antibiotic resistance among the aquaculturists is the urgent need of the hour as a precaution and preventive steps against the use of antibiotics.

## CRediT authorship contribution statement

R.K.N. Contributed in conceptualization, sample collection, carrying out experimental study, writing of original draft.

S.K.P. Contributed in conceptualization, experimental design and interpretation of results

M.R.B. Contributed in sampling design, sample collection and manuscript preparation.

P.P.K. Contributed in arranging laboratory facilities, editing and conducted the statistical analysis

R.R.P. Contributed in conceptualization and supervised the research

M.P.M. contributed in conceptualization, sampling design, Institute fund acquisition, editing and overall supervision of the study.

## Declaration of competing interest

We do declare that no conflict of interest in financial aspects and no personal relationships in publishing this research work.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2021.112887.

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