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High prevalence of Aeromonas and Pseudomonas infections among cage cultured Pangas catfish from the reservoirs of Maharashtra, India

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Key words: Cage culture; Pangasius catfish; Bacterial infection; Histopathology; Principal Component Analysis

Abstract

Cage farming of Pangas catfish (*Pangasionodon hypophthalmus)* in small reservoirs is primarily profitable. Still, sustainability in terms of disease and environmental impact issues remains unsolved. In the present study, Pangas catfish from selected farms were screened for bacteria and parasites from 2017-18. In the present study, several Gram-negative bacterial species, including *Edwardsiella tarda*, *Aeromonas hydrophila, A. veronii, Pseudomonas putida, Enterobacter cloacae,* and *Plesiomonas shigelloides*, were isolated. A high prevalence of Aeromonas and Pseudomonas infections was noticed in cage farms. The most prevalent isolate was *A. veronii* (9 isolates), followed by *P. aeruginosa* and *P. putida*, revealing the presence of biotic stress.

Furthermore, potential human pathogenic bacteria, particularly *Klebsiella pneumoniae, P. aeruginosa, Acinetobacter baumannii, Citrobacter freundii,* and *Morganella morganii*, were isolated. Histopathological analysis of the vital organs concluded the extent of damage caused due to the biotic stress in the cage culture system. The antibiotic sensitivity test implied resistance of bacteria for Sulphamethoxazole and Tetracycline. Some of them were resistant to multiple drugs, the risk for public health. Water temperature, dissolved oxygen, pH, Aeromonas, Pseudomonas, and Plesiomonas were identified by principal component analysis as significant abiotic and biotic stress factors. This information helps design predictive disease models.

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Introduction

Cage culture of Pangas catfish *(Pangasianodon hypophthalmus)* in India was started in 2012. The high stocking density and better growth rate make it suitable for cage farming in small and large reservoirs. However, the frequency of disease outbreaks is more in cage cultured fish than in ponds (Collins, 1988; Masser, 1991). Poor water quality parameters and improper management practices such as high stocking, overfeeding, inadequate nutrition, etc. also increase stress to the fish and thus make them more susceptible to disease outbreaks (Boyd & Tucker, 1998; Zamri-Saad et al. 2014). Reports on cage cultured Pangas catfish diseases from Indian water bodies are infrequent (Kumar et al. 2013; 2015), whereas many of cases have been reported from Vietnam. Most of the diseases are due to bacteria namely, *A. hydrophila A. sobria*, and *A. caviae, A. jandaei* (Ferguson et al. 2001; Crumlish et al. 2001; Kumar et al. 2015) causing Motile Aeromonad Septicemia (MAS) and *Edwardsiella ictaluri* causing Bacillary Necrosis of Pangasius (BNP). However, many prevalent bacteria and parasites, i.e., Aeromonas, Pseudomonas, Streptococcus (Austin & Austin, 2012; Amal et al. 2013; Chitmanat et al. 2016), Edwardsiella (Phan et al. 2009; Shetty et al. 2014), Trichodina (Chitmanat et al. 2016) and Myxosoma are reported to cause diseases in different cage cultured fishes.

Abiotic factors associated with the aquaculture system can significantly affect the health of the cultured animal. In an open water culture system, water quality fluctuation is higher influencing the bacterial diversity (Vezzulli et al. 2002). The optimal range of water quality parameters varies from species to species and should be monitored to achieve better growth and survival. Therefore, it is considered as a critical factor in the aquafarming industry. Despite massive Pangasius farming, some reports deal with their disease predictive model and management strategies (NFDB, 2016). In this context, the present research was carried out to understand the prevalence of the bacterial pathogens in the cage cultured *P. hypophthalmus* in selected reservoirs of Maharashtra (Panshet, Varasgaon, Kanher, and Manoli).

Furthermore, to find significant biotic and abiotic factors associated with the cage culture of pangasius catfish, Principal Component Analysis (PCA) was also carried out. The periodic data of prevalent pathogens would be helpful for disease prediction and control. Antimicrobial resistance is a burning topic for humans, and thus have also carried out antibiotic sensitivity tests to assist with cage culture bacterial pathogens.

Materials and Methods

Details of sampling

The sampling was conducted periodically from September 2017 to May 2018 in cage farms situated in various reservoirs of Maharashtra (**Figure 1**), i.e., Farm I (Panshet), Farm II (Varasgaon), Farm III (Kanher), and Farm IV (Manoli). The details of reservoirs and cages are mentioned in **Table 1**. Two stations were fixed in each cage for the collection of water and soil samples. The water samples were taken from various points at each cage and mixed to get a representative sample. Water quality parameters namely water temperature, pH, dissolved oxygen, ammonia-N, and nitrite-N were analyzed using APHA (2005). A total of 20 fishes, both healthy and diseased, were randomly collected from each reservoir in every sampling with an average size of 215g-450g. Some of them exhibited petechial hemorrhagic lesions on the body surface and pectoral-fin base, reddening of mouth, abnormal swimming pattern, lethargic movement, and tail rot (**Figure 2**). The fishes were brought to the laboratory in live condition for further analysis.

Bacterial and parasitic examination

External and internal organs, i.e., skin mucus, gill, fin, kidney, liver, and gall bladder were observed under the microscope for external and internal parasitic examination. Fishes were surface sterilized with 70% alcohol to avoid any unwanted bacterial contamination. A sterilized loop was inserted into the external lesions, kidney, liver, and gill and aseptically streaked on Brain heart infusion agar and incubated for 18-24 hr at 25-28^{0}C. Isolated colonies were further streaked on selective agar, i.e., Aeromonas isolation agar, SS agar, and GSP agar. The pure culture was used for biochemical and molecular characterization. Presumptive biochemical tests such as gram staining, oxidase, and catalase were done

using standard procedures, and further characterization was done using the biochemical identification kit (HiMedia Ltd, Mumbai).

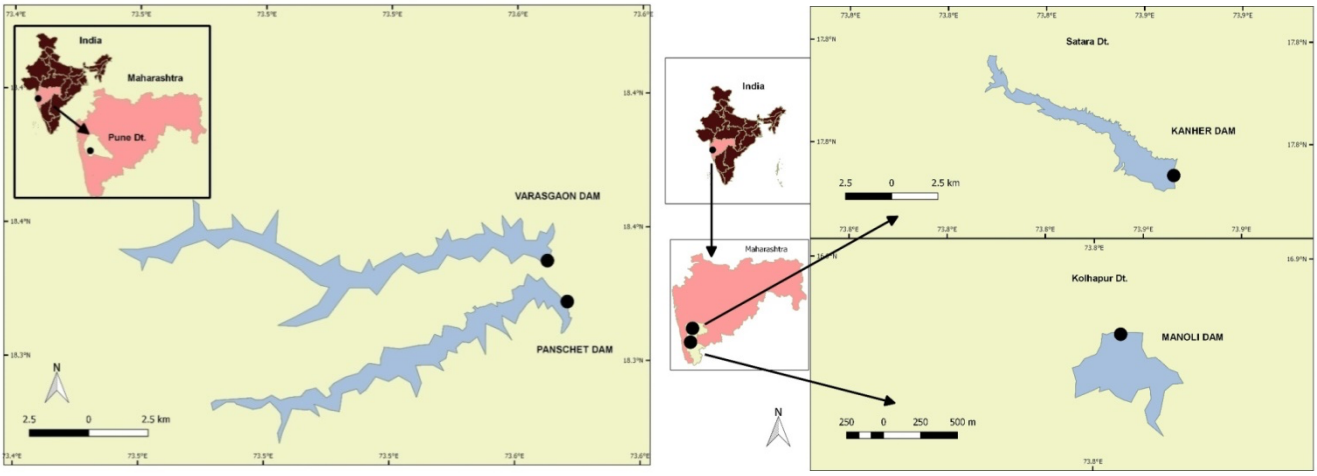

Figure 1 Sampling Location of the present study in Maharashtra.

Table 1 Characteristics of selected reservoirs and cage farms

Cage farms	*Farm I*	*Farm II*	*Farm III*	*Farm IV*
Location of cages	18° 22' 24.7296'' N 73° 36' 4.2408'' E	18° 23' 23.0784'' N 73° 35' 54.9312'' E	16° 37'41.3508'' N 74°16' 48.4896'' E	16° 56' 52. 7604'' N 73° 48'28.6776'' E
Name of the reservoir	Panshet	Varasgaon	Kanher	Manoli
Area of the reservoirs	870ha	1180ha	350ha	300 ha
Depth of the reservoirs	48m	50m	16m	18m
Number of cages	50 (5 x 5 x3 .5 m)	150 (5x 5 x 3.5 m)	8 (6 x 7 x 4m)	48 (4 x 6 x 5m)
Stocking density	6500/cage wt. 25-30 g	6500/cage wt. 25-30 g	6000/cage wt. 20-25 g	4000/cage wt. 20-25 g

Figure 2 The fish samples collected for bacteriological analysis displaying clinical symptoms

Molecular characterization of the isolates

Bacterial DNA isolation was done using the CTAB method and quantified using Nanodrop2000 (Thermo-scientific, USA). Molecular identification of the bacterial isolates was carried out by 16s rRNA gene sequencing, using universal 16s rRNA primers. The forward and reverse primer sequences were 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-GGTTACCTTGTTACGACTT-3' respectively (Sarkar et al. 2012). PCR protocol was performed by using 25µl of a PCR mixture containing 2µl of 50ng DNA template, 1µl of 10 pmol of each specific primer, 1µl of 200 µM dNTPs, 0.5µl of Taq DNA polymerase, and 2.5µl of 1x Taq polymerase buffer containing 1.5 mM $MgCl_2$. PCR amplification was carried out with the following temperature program: 1 cycle of denaturation for 5 min at 95°C; 35 cycles of melting at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1min; and a final extension at 72°C for 10 min and then held at 4°C. PCR amplicons were separated electrophoretically by loading PCR product in 1.5% agarose gel. The target amplicon bands were purified using the StrataPrep DNA Gel Extraction Kit (Agilent Technologies, USA), following the manufacturer's protocol. The purified 16s rRNA gene amplicons of the bacterial isolates were sequenced using commercial services of Xcelris Labs Ltd. (Bangalore). The single nucleotide-nucleotide alignment was performed to find the homologous sequence using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) (**Figure 3**).

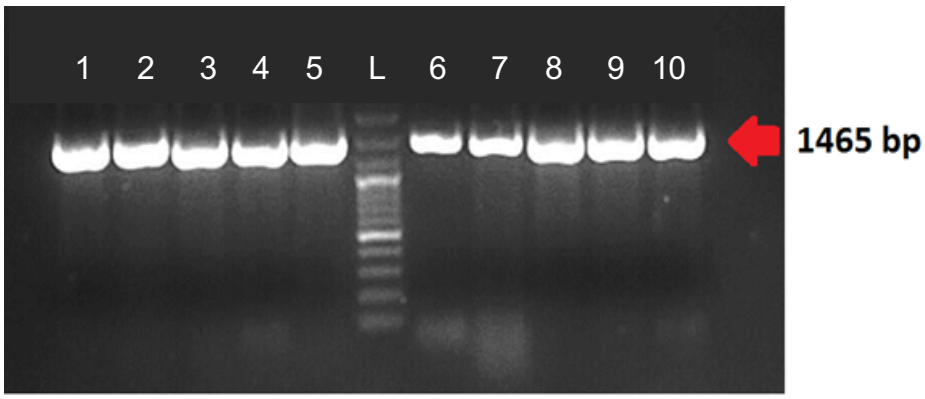

Figure 3 16s rRNA gene PCR amplification of the isolated bacterial pathogens using universal 16s rRNA primers (Sarkar et al., 2012) displaying distinct bands at 1465 bp. Lane L: 100bp plus ladder (Image analyzed by Gel Doc System, 2000, Biorad).

Histopathological analysis

Histopathological analysis for the vital tissues of fishes isolated with *Aeromonas, Pseudomonas sp* as well as healthy fishes was carried out to compare tissue level alterations. The vital tissues (liver, kidney, gill, and intestine) were collected and preserved in 10% neutral buffered formalin for histopathological analysis. Tissues were dehydrated with a series of alcohol gradients and cleared by using xylene. The dehydrated tissues were embedded in paraffin wax following the impregnation technique (Leica, EG 1140H, Germany). The embedded wax blocks were sectioned at 3-5 µm thickness using a microtome (Leica RM 2125RT, Germany) and stained with hematoxylin and eosin (Luna, 1968).

Antibiotic susceptibility

Disc diffusion method, also called as Kirby-Bauer test was carried out for screening sensitive and resistant bacteria against 10 different antibiotics namely, Ampicillin (25mcg), Tetracycline (10 mcg), Ciprofloxacin (30 mcg), Gentamicin (50 mcg), Sulphamethoxazole (25 mcg), Kanamycin (5 mcg), Streptomycin (25 mcg), Chloramphenicol (30mcg), Penicillin – G (2 units) and Neomycin (30 mcg). The 24 h fresh bacterial culture was spread over Mueller Hinton agar plates. The antibiotic discs were placed aseptically on agar and incubated for 24 h at 25ºC. The presence or absence of an inhibitory area around the disc was measured in mm and the results were recorded. The zone sizes were compared and isolates were classified as sensitive, resistant, or intermediate according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2009).

Water quality parameter analysis

Two stations were fixed in each cage for the collection of water samples. Water samples were taken from various points in each cage and mixed to get a representative sample. Water quality parameters such as water temperature, pH, dissolved oxygen, ammonia-N, and nitrite-N were analyzed by using APHA (2005).

Statistical analysis

Statistical analysis of mean ± SD of water quality data was measured and compared between each farm and samplings using one-way ANOVA with Duncan's multiple range test (Duncan, 1955) through SPSS 16.0. The number of bacteria isolated from each farm and their percentage during the study period was calculated. P-value at <0.05 was used to specify statistical significance. A dataset was prepared with the percentage of all isolated bacteria and all water quality data in each sampling and analyzed separately for each cage farm. Parameters that exhibited significant variation in their values were computed in MS Excel for further analysis using PAleontological STatistics (PAST) software package. The association between various water quality parameters in the relevance of bacterial load was studied using Principal Component Analysis (PCA). The PCA was examined using PAST

Software (Version 3.1.0). The summary statistics explained with minimum-maximum range, standard deviation, and variance for various water quality parameters and bacterial prevalence were calculated separately for each farm and used for further analysis.

Results

Isolation and identification of bacteria

A total of 21 bacterial species were identified and characterized by using 16s rRNA gene sequencing (Fig. 3). The identified bacteria were *Pseudomonas sp* (6 nos), *Aeromonas sp* (3 nos), *Acinetobacter sp* (3 nos), *Citrobacter sp* (2 nos), *Enterobacter sp* (2 nos), *P. shigelloides*, *Klebsiella pneumonia, Morganella morganii, Lactococcus lactis,* and *E. tarda*. The 16s rRNA sequences were successfully submitted to the NCBI Bankit database (**Table 2**). Among all bacteria, *A. veronii* was found to be the most prevalent (9 nos) followed by *P. aeruginosa* (7 nos).

Table 2 List of bacterial pathogens isolated from various cage farms and their NCBI accession numbers.

Cage farms	*Name of the bacterial isolate*	*Accession ID*	*Cage farms*	*Name of the bacterial isolate*	*Accession ID*
Farm I	*Plesiomonas shigelloides*	MK038969	Farm III	*Pseudomonas aeruginosa*	MK045608
	Lactococcus lactis	MK026803		*Pseudomonas aeruginosa*	MK045611
	Aeromonas veronii	MK027224		*Acinetobacter sp.*	MK045613
	Pseudomonas aeruginosa	MK026805		*Acinetobacter baumannii*	MK045614
	Aeromonas veronii	MK038967		*Pseudomonas sp.*	MK045615
	Pseudomonas aeruginosa	MK038970		*Pseudomonas mosselii*	MK045617
	Citrobacter youngae	MK027057		*Enterobacter cloacae*	MK045618
Farm II	*Morganella morganii*	MK027059		*Pseudomonas entomophila*	MK045807
	Plesiomonas shigelloides	MK038971		*Pseudomonas entomophila*	MK027248
	Aeromonas hydrophila	MK038972		*Enterobacter asburiae*	MK045607
	Edwardsiella tarda	MK038966		*Acinetobacter calcoaceticus*	MK905213
	Aeromonas hydrophila	MK044848		*Aeromonas jandaei*	MK905214
	Acinetobacter baumannii	MK044849		*Citrobacter freundii*	MK050539
	Plesiomonas shigelloides	MK045606		*Pseudomonas entomophila*	MK045812
	Aeromonas veronii	MK044839		*Pseudomonas putida*	MK045810
	Aeromonas veronii	MK044845		*Pseudomonas putida*	MK050534
	Pseudomonas aeruginosa	MK045621		*Pseudomonas putida*	MK050535
	Aeromonas veronii	MK045622		*Pseudomonas putida*	MK050536
	Acinetobacter baumannii	MK027249	Farm IV	*Aeromonas veronii*	MK077636
	Klebsiella pneumoniae	MK905218		*Pseudomonas aeruginosa*	MK078036
Farm III	*Aeromonas veronii*	MK050532		*Enterobacter cloacae*	MK078043
	Aeromonas veronii	MK050537		*Enterobacter asburiae*	MK078044
	Klebsiella pneumoniae	MK027254		*Pseudomonas putida*	MK078045
	Aeromonas veronii	MK007964		*Pseudomonas aeruginosa*	MK078046
	Pseudomonas stutzeri	MK050538		*Pseudomonas sp.*	MK078630
				Acinetobacter calcoaceticus	MK079615

Physicochemical parameters of water and soil

The water and soil quality analysis (**Table 3**) reveals that most of the parameters were in the normal range except for less dissolved oxygen (4.85 ppm) and high ammonia (0.3 ppm) in the Manoli reservoir.

Prevalence of bacterial infections in cage farms

A total of 240 fishes were examined for bacterial infections from all the farms in which 46.6% of fishes were infected with bacteria**.** Aeromonas and Pseudomonas are the most prevalent bacterial infections noticed in all the cage farms whereas other bacterial infections were comparatively less. The Pseudomonas species isolated were *P. aeruginosa, P. putida, P. entomophila, P. mosselli,* and *P. stutzeri. A. hydrophila, A. veronii,* and *A. jandaei* were the Aeromonas species isolated from cage cultured fishes. Prevalence of

bacterial infections was 14%, 22%, 42%, and 34% in cage farms I, II, III, and IV respectively (**Table 3 & 4**).

Table 3 Prevalence of major bacterial infections from cage farms I & II

Farms	*Farm I*			*Farm II*		
Bacterial group	*No. of fishes examined*	*No. of fishes Infected*	*Prevalence (%)*	*No. of fishes examined*	*No. of fishes Infected*	*Prevalence (%)*
Aeromonas		*7*	*11.6*		*11*	*18.3*
Pseudomonas		*4*	*6.6*		*-*	*-*
Acinetobacter		*-*	*-*		*4*	*6.6*
Citrobacter	*60*	*-*	*-*	*60*	*2*	*3.3*
Enterobacter		*-*	*-*		*-*	*-*
Pleisomonas		*2*	*3.3*		*2*	*3.3*
Other infections		*1*	*1.6*		*3*	*5*
Total	*60*	*14*	*23*	*60*	*22*	*36.6*

Table 4 Prevalence of major bacterial infections from cage farms III & IV

Farms	*Farm III*			*Farm IV*		
Bacterial group	*No. of fishes examined*	*No. of fishes Infected*	*Prevalence (%)*	*No. of fishes examined*	*No. of fishes Infected*	*Prevalence (%)*
Aeromonas		11	18.3		8	13.3
Pseudomonas		19	31.6		20	33.3
Acinetobacter		6	10		3	5
Citrobacter	60	-		60	1	1.6
Enterobacter		4	6.6		2	3.3
Pleisomonas		-			-	-
Other infections		2	3.3		-	-
Total	60	42	70	60	34	57

Antibiotic susceptibility

Antibiotic susceptibility tests indicated that all the bacterial isolates were sensitive to Gentamicin and Ciprofloxacin and most of the isolates were resistant to Ampicillin and Penicillin-G. The percentage of resistant bacteria was 32% and 28% for Sulphamethoxazole and Tetracycline respectively (**Table 5**). An intermediate level of resistance was noted in the case of aminoglycoside antibiotics, i.e., Neomycin, Kanamycin, and Streptomycin.

Histopathological analysis

Significant changes were noticed in the fishes with a high prevalence of Pseudomonas and Aeromonas infections when compared with the control fishes (**Figure 4 & 5**). The following alterations observed in the fishes infected with Pseudomonas. Secondary lamellar fusion, epithelial hyperplasia, infiltration of inflammatory cells, curling and necrosis in gill tissue; pyknotic nuclei, congestion, focal area of cellular infiltration, atrophied pancreatic tissue and necrotized hepatocytes in the liver parenchyma; tubular necrosis, infiltration of inflammatory cells, congestion, hypertrophy of tubular epithelial cells with the consequent reduction of tubular lumen were noticed in kidney tissue. Histopathological alterations seen in Aeromonas infected fishes are, secondary lamellar fusion, hyperplasia, curling, atrophy, and necrosis in gill tissue; pyknotic nuclei, vacuolation, hemorrhage, and congestion in the

liver parenchyma; hemorrhages, tubular necrosis, and hypertrophy of tubular epithelial cells with the consequent reduction of tubular lumen were observed in kidney tissue.

Table 5 Antibiotic susceptibility profile of bacterial isolates

Sl/no	*Name of the Antibiotics*	*Resistance (%)*	*Intermediate (%)*	*Susceptible (%)*
1.	Chloramphenicol (30 mcg)	8	4	88
2.	Kanamycin (5 mcg)	4	40	56
3.	Ampicillin (25 mcg)	96	-	4
4.	Sulphamethoxazole (25 mcg)	32	4	64
5.	Neomycin (30 mcg)	4	60	36
6.	Tetracycline (10 mcg)	28	24	48
7.	Penicillin – G (2 units)	80	-	20
8.	Streptomycin (25mcg)	12	44	44
9.	Gentamicin (50mcg)	-	4	96
10.	Ciprofloxacin (30mcg)	-	16	84

*mcg – micrograms

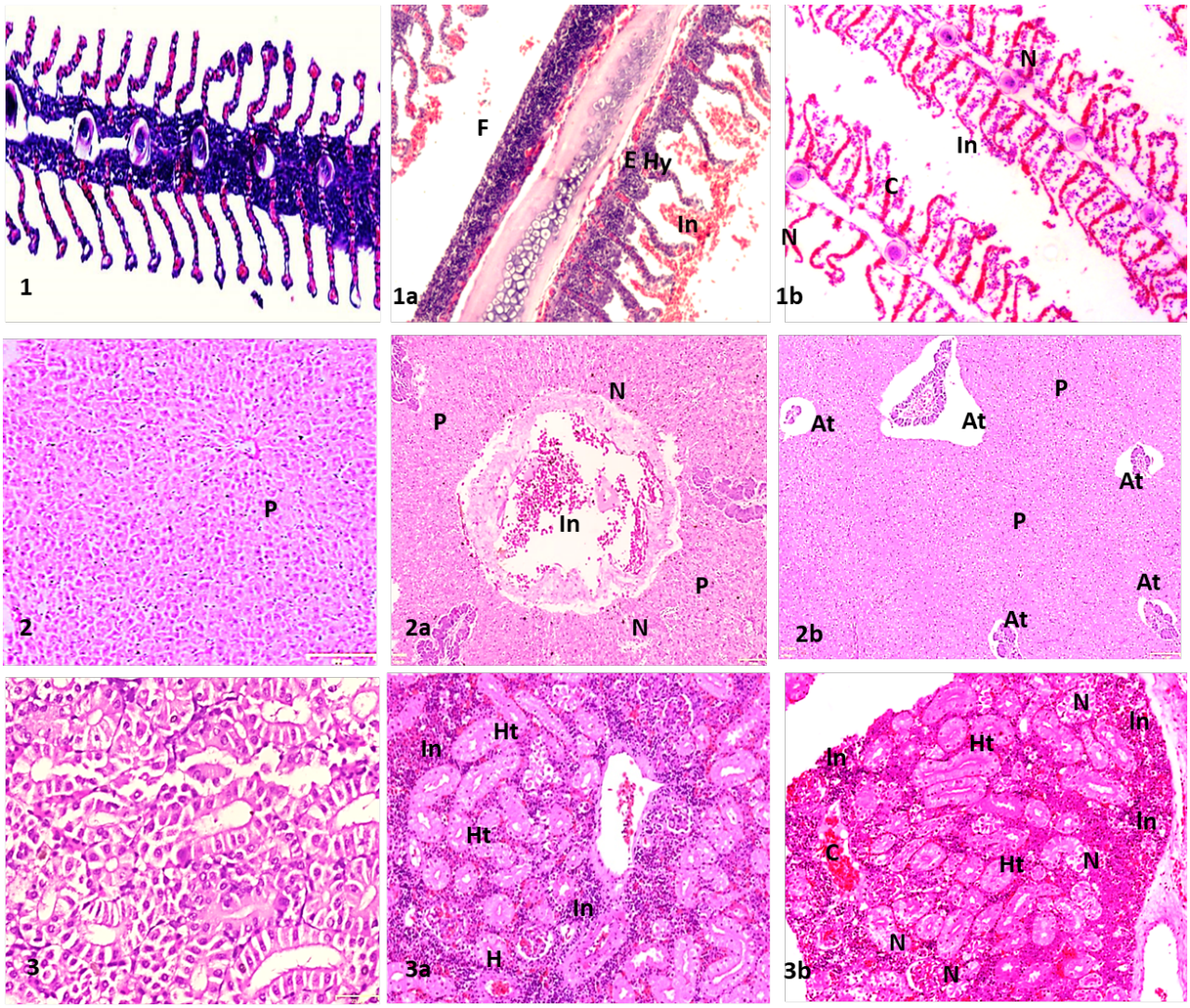

Figure 4 Comparison of the histopathological alterations observed in the vital tissues of fishes infected with Pseudomonas sp. and the healthy fishes. 1) Gill tissue section displaying normal secondary lamella; 1a) Gill tissue displaying epithelial hyperplasia (E hy), infiltration of inflammatory cells (In) and lamellar fusion (F); 1b) Gill section exhibiting curling of secondary lamellae (C), infiltration of inflammatory cells (In) and Necrosis at

places (N). 2) Liver tissue section exhibiting normal hepatocytes; 2a) Liver tissue exhibiting degenerative changes i.e., necrotized hepatic cells (N), pyknotic nuclei (P) and focal area of cellular infiltration (In); 2a) Liver section displaying congestion at hepatic vessels (C) and pyknotic nuclei (P). 3) Kidney tissue with normal renal tubules; 3a) Kidney tissue displaying degenerative changes i.e., hypertrophy of tubular epithelial cells with consequent reduction in the tubular lumen (Ht) and Infiltration of inflammatory cells in the kidney parenchyma (In); 3b) Kidney section exhibiting tubular necrosis (N) and haemorrhage at places (H).

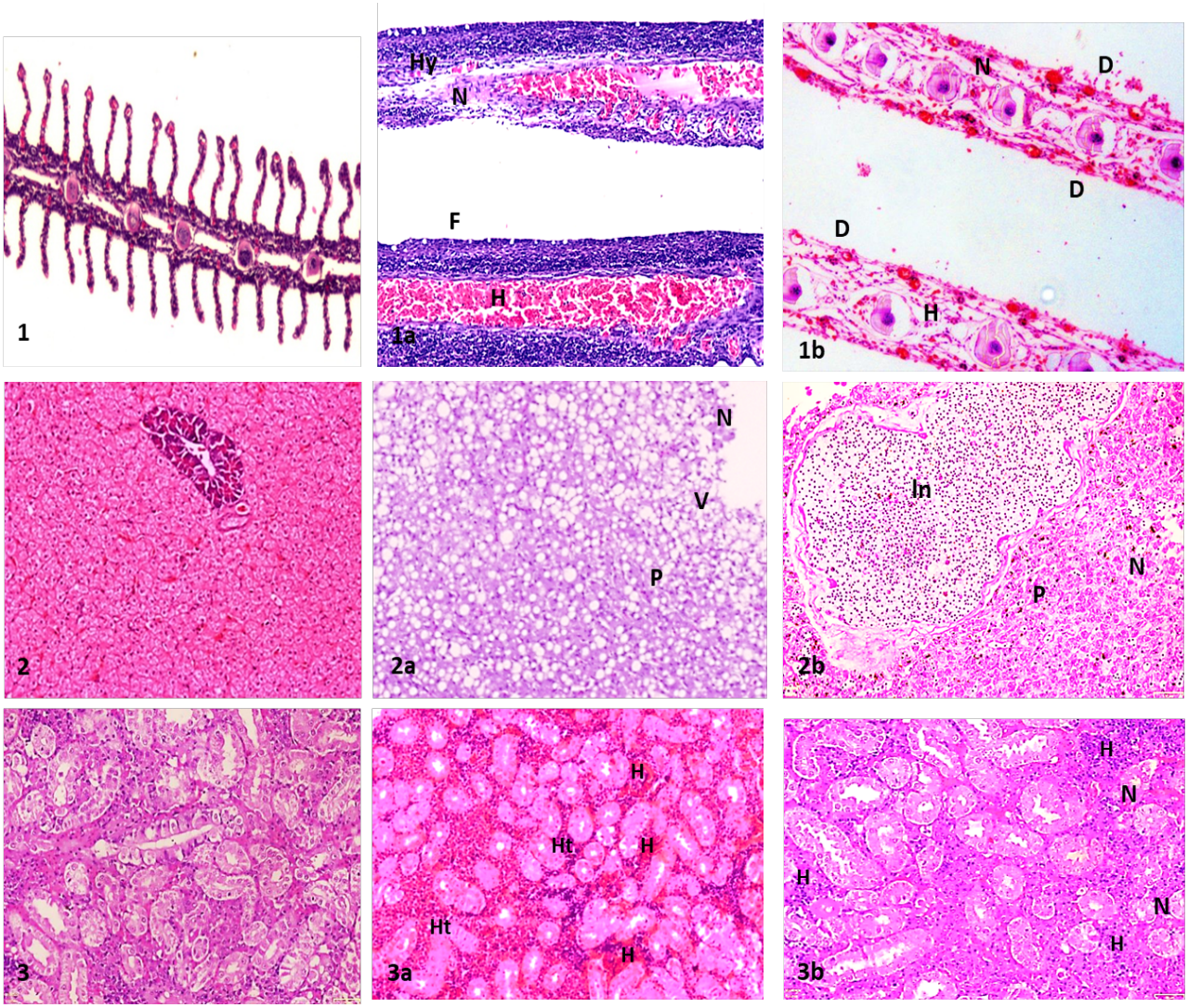

Figure 5 Comparison of the histopathological alterations observed in the vital tissues of fishes infected with Aeromonas sp. and the healthy fishes. 1) Gill tissue section displaying normal secondary lamella; 1a) Gill tissue displaying hyperplasia (Hy) with consequent complete fusion of secondary lamellae (F), necrosis (N) and haemorrhage (H) at places; 1b) Gill section exhibiting haemorrhage (H), necrosis of gill tissue (N) and complete degradation of secondary lamellae (D). 2) Liver tissue section exhibiting normal hepatocytes; 2a) Liver tissue exhibiting necrotized hepatic cells (N), pyknotic nuclei (P) and focal area of cellular infiltration (In); 2a) Liver section displaying congestion at hepatic vessels (C) and pyknotic nuclei (P). 3) Kidney tissue with normal renal tubules; 3a) Kidney tissue displaying degenerative changes i.e., hypertrophy of tubular epithelial cells with consequent reduction in the tubular lumen (Ht) and Infiltration of inflammatory cells in the kidney parenchyma (In); 3b) Kidney section exhibiting tubular necrosis (N) and haemorrhage at places (H).

Principal component analysis

Principal component loadings (PC_1 and PC_2) of each farm were obtained after principal component analysis of biotic factors and water quality parameters (**Table 7**). Two axes were formed for each farm in PCA and their cumulative percentage explained was 99.92%, 99.80%, 99.53, and 99.81% of water quality variations in Farm I, Farm II, Farm III, and Farm IV respectively. In Farm I, PC_1 had positive loadings of water temperature, dissolved oxygen, and pH while in PC_2 pH, the prevalence of Aeromonas, Pseudomonas, and Pleisomonas had high loadings (**Figure 6**). In the case of Farm II, the first axis positive loadings were similar to Farm I whereas the second axis had high loadings of pH, the prevalence of Citrobacter and Pleisomonas (**Figure 7**). However, for Farm III, water temperature, dissolved oxygen, pH, and the prevalence of Pseudomonas were found to show high loadings in PC_1 and phosphate, prevalence of Aeromonas, Pseudomonas and Enterobacter were the high loadings in PC_2 (**Figure 8**). For Farm IV, PC_1 had high loadings of water temperature, pH, and the prevalence of Pseudomonas while in PC_2 water temperature, dissolved oxygen, pH, nitrite, nitrate, the prevalence of Pleisomonas and other infections were shown high loadings (**Figure 9**). Overall, significant principal loadings observed are water temperature, dissolved oxygen, and pH among the water quality parameters and the prevalence of Pseudomonas, Aeromonas and Pleisomonas among the biotic stress factors identified in this study.

Table 7 Principal component loadings from principal component analysis of water quality and bacterial parameters from all the farms

Parameters	*Farm - I*		*Farm - II*		*Farm - III*		*Farm - IV*	
	PC-I	*PC-II*	*PC-I*	*PC-II*	*PC-I*	*PC-II*	*PC-I*	*PC-II*
Percentage variance	99.712	0.213	99.376	0.431	96.936	2.598	95.992	3.818
Cumulative variance	99.712	99.925	99.376	99.807	96.936	99.534	95.992	99.810
Eigen value	2.991	0.006	2.981	0.013	2.908	0.078	141.867	5.643
Water temperature	**3.276**	-0.242	**3.264**	0.050	**3.191**	-0.408	**3.170**	**0.436**
Dissolved oxygen	**0.411**	-0.305	**0.436**	0.181	**0.345**	-0.626	0.284	**1.085**
pH	**0.577**	**0.358**	**0.548**	**0.765**	**0.608**	-0.813	**0.642**	**0.821**
Nitrite	-0.450	-0.481	-0.480	0.156	-0.563	-0.398	-0.541	**0.309**
Ammonia	-0.428	-0.352	-0.451	0.091	-0.554	-0.383	-0.521	0.256
Nitrate	-0.419	-0.331	-0.446	-0.212	-0.561	-0.412	-0.538	**0.318**
Phosphate	-0.444	-0.404	-0.472	0.122	-0.445	**0.310**	-0.425	-0.207
Aeromonas	-0.126	**1.299**	0.020	-2.121	-0.050	**0.757**	-0.136	-0.668
Pseudomonas	-0.266	**1.123**	-0.486	0.209	**0.302**	**3.137**	**0.475**	-3.153
Acinetobacter	-0.456	-0.596	-0.309	-0.812	-0.265	-0.431	-0.389	-0.080
Citrobacter	-0.456	-0.596	-0.395	**0.534**	-0.568	-0.415	-0.491	0.0005
Enterobacter	-0.456	-0.596	-0.486	0.209	-0.394	**0.411**	-0.442	0.240
Pleisomonas	-0.355	**2.492**	-0.390	**2.205**	-0.568	-0.415	-0.545	**0.321**
Other infections	-0.409	-1.368	-0.352	-1.377	-0.478	-0.314	-0.545	**0.321**

*Water quality loadings with absolute value >0.30 in bold.

Farms	*PC 1*	*PC 2*
Farm I	0.81718	-1.0341
Farm II	0.91287	1.0045
Farm III	-0.89916	0.69341
Farm IV	-0.83089	-0.66381

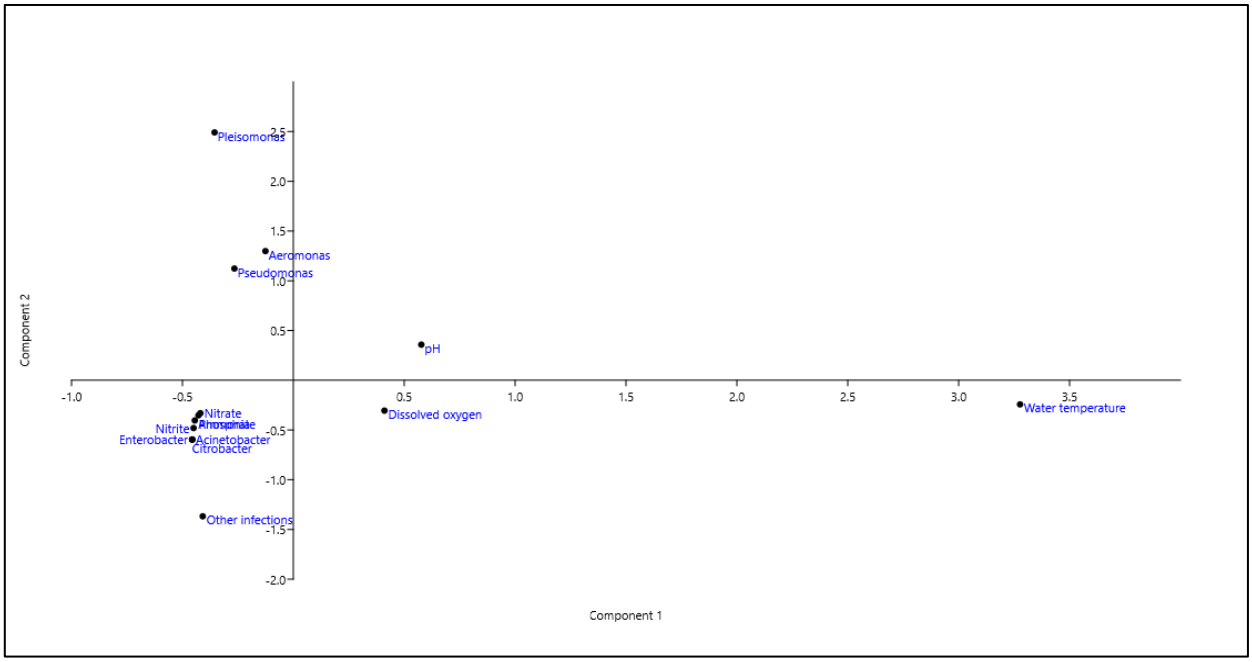

Figure 6 Principal component analysis ordination diagram showing the significant biotic and abiotic factors' loadings associated with Farm I.

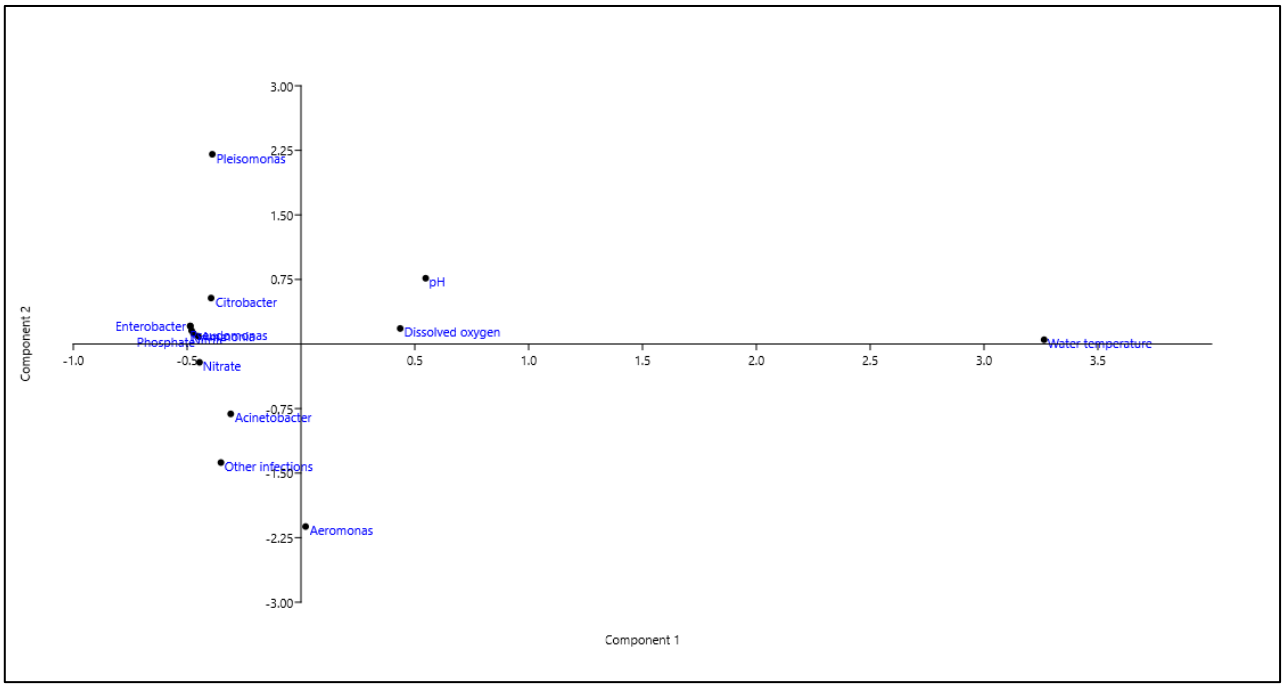

Figure 7 Principal component analysis ordination diagram showing the significant biotic and abiotic stress factors' loadings associated with Farm II.

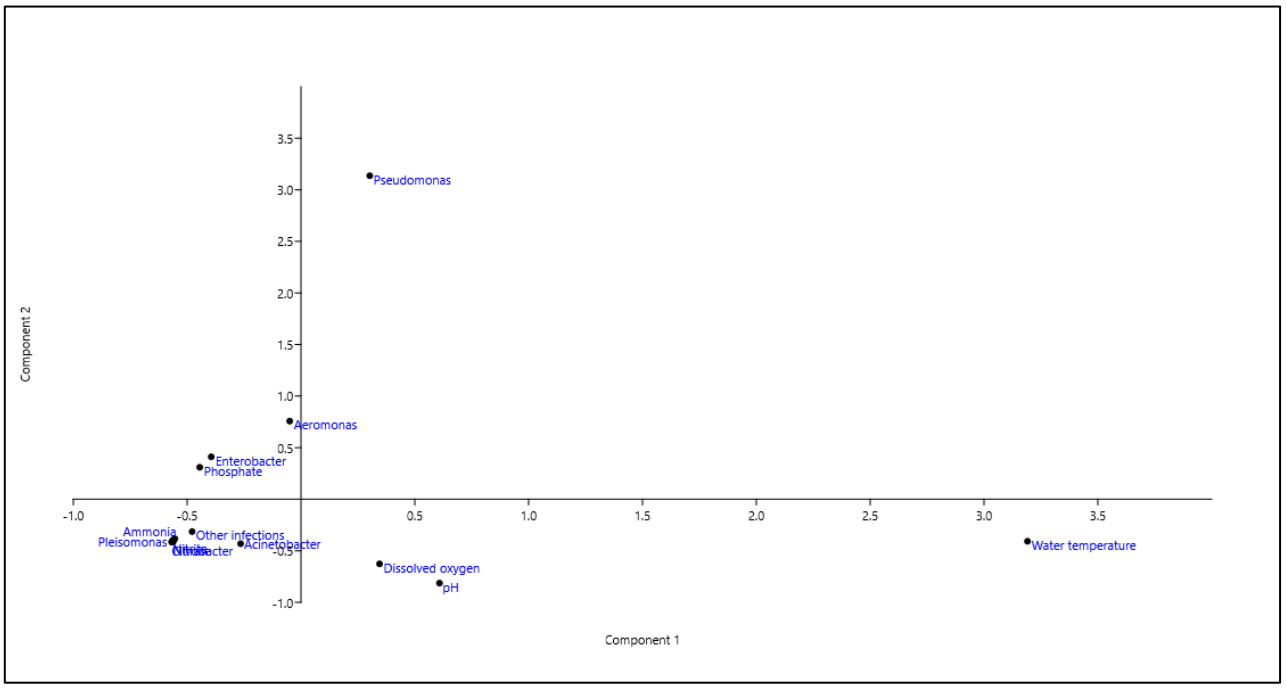

Figure 8 Principal component analysis ordination diagram showing the significant biotic and abiotic stress factors' loadings associated with Farm III.

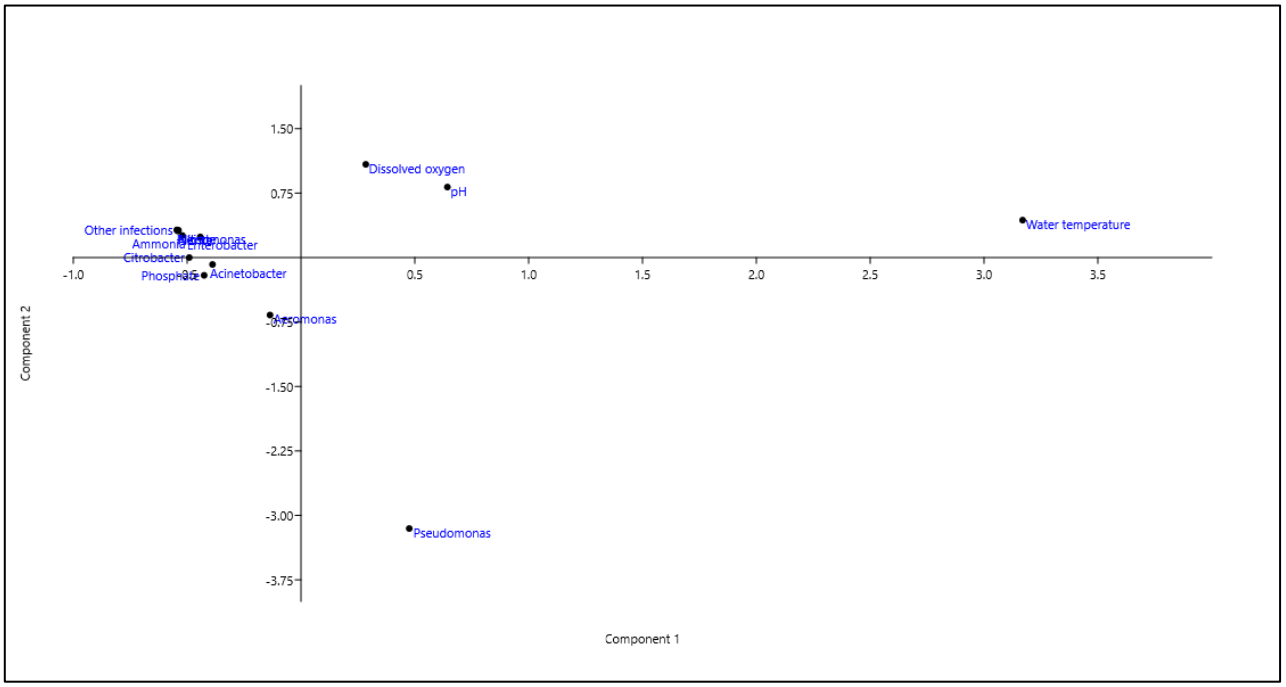

Figure 9 Principal component analysis ordination diagram showing the significant biotic and abiotic stress factors' loadings associated with Farm IV.

Discussion

Cage culture is one of the most intense forms of aquaculture and can be prone to disease problems. The natural as well as artificial environmental stress factors have a significant impact on fish health. It has also been reported that acute or chronic exposure to these factors may lead to many infectious disease outbreaks in fishes (Bly *et al*., 1997). Hence the study was formulated to understand the prevalence of microbes in cage cultured Pangas catfish.

Most of the fish bacterial diseases are caused by gram negative opportunistic bacteria which are generally adapted to the low osmotic condition of the aquatic environment (Austin & Austin, 2016; Tesfaye et al. 2018). There were nine gram-negative bacteria isolated, whereas *Lactococcus* is the only gram-positive bacteria found in the present study. Overall, 12 *Aeromonas* sp, comprising 3 species, i.e., hydrophila, veronii, and jandaei were isolated and characterized during the present investigation. *A. hydrophila* is responsible for heavy mortality and eliciting septicemic conditions in the Pangas catfishes (Sarkar et al. 2016; Nahar et al. 2016). In fact, Nahar et al. (2016) had isolated 10 virulent strains of *A. hydrophila* from farmed juvenile pangas catfish. Kumar et al. (2015) has isolated a virulent strain of *A. jandaei* from Pangas catfish with the clinical manifestations of reddish lesions near the pectoral fins and belly region. According to our results, *A. veronii* is the most prevalent bacteria isolated. However, information regarding disease outbreaks associated with *A. veronii* in Pangas catfish is not well reported. Besides, *A. veronii* was stated as a causative agent for hemorrhagic septicemia in various fish species (Sreedharan et al. 2013; Eissa et al. 2015; Hassan et al. 2017).

During our study, *E. tarda* was isolated from the kidney of Pangas catfish with the reddish lesion in the mouth and fins without any significant mortality of fishes in the cages. Similar findings were acquired by Shetty et al. (2014), who isolated *E. tarda* from the clinically manifested Pangas catfish with no mortality but they could reciprocate the clinical signs during the experimental infection. From the present study, *Lactococcus* sp identified without any obvious clinical signs which are previously known as probiotic bacteria (Balcazar et al. 2008). Recently it has been reported as a causative agent of Lactococcosis, responsible for a 100% loss of hybrid sturgeons (*Huso huso* × *Acipenser ruthenus*) in a fish farm in Taiwan, China (Chen et al. 2012).

A highly diverse group of Pseudomonas comprising species namely, *P. entomophila, P. putida, P. aeruginosa, P. mosselii, and P. stutzeri* were identified in different reservoirs. *P. aeruginosa* is well known as an opportunistic pathogen for *Oreochromis mossambicus* (Thomas et al. 2014) and *Clarias gariepinus* (Khairnar et al. 2013) but it is not yet reported as a disease-causing bacterium in Pangasius. Out of two species of Acinetobacter (*A. baumannii, A. calcoaceticus*) identified from the present investigation, *A. baumannii* is well reported as a multi-drug resistant pathogen associated with the eye infection of *Channa striatus* in India (Rauta et al. 2011). However, Reddy and Mastan (2013) have reported *A. schindleri* associated with red-eye infection of *Pangasius sutchi*. *P. shigelloides* identified from our study is a pathogenic bacterium to *Ctenopharyngodon idella* (Hu et al. 2014), *O. nilotica* (Nisha et al. 2014; Liu et al. 2015), and *Hypophthalmichthys molitrix* (Behera et al. 2018).

Some of the human bacterial pathogens were isolated namely *C. youngae, A. baumannii, K. pneumoniae, M. morganii, A. calcoaceticus, P. shigelloides* (enteric disease), and *C. freundii* from this study (Falagas et al. 2006; McConnell et al. 2013). These are mostly associated with food-borne zoonotic diseases and multi-drug resistance issues in humans. Isolation of such a high number of zoonotic bacterial pathogens from the open water culture system increases the public health concern associated with farmed fish consumption (Cantas and Suer, 2014).

Multivariate analyses displayed that water temperature, dissolved oxygen, pH, phosphate, nitrite, and nitrate were vital in at least one of the farms that were associated with the prevalence of bacteria. Similar results were observed by Amal et al. 2013 in cage cultured red hybrid tilapia. They reported that water temperature, dissolved oxygen, pH, and ammonia exhibited a notable correlation with the presence of *Streptococcus sp* in the cage

culture system. Unfavorable water quality is well discussed as a significant stress factor, influencing the presence of pathogenic and non-pathogenic bacterial communities in aquaculture systems (Amal et al. 2013; Ismail et al. 2016). Apart from influencing the host immune system, the environmental factors also significantly alter the pathogen life cycle, abundance, and host range (Schade et al. 2015). Karvonen et al. (2010) has reported that the increased water temperature has a dynamic influence on the prevalence of bacterial and parasitic fish diseases. According to the investigation by several authors, the intensity of gram-negative opportunistic bacteria such as *P. aeruginosa*, *Escherichia coli, A. baumannii,* and *Enterobacter cloacae* (Psoter et al. 2013; Schwab et al. 2015) have increased during summer as compared to gram positive bacteria. Our research findings also in agreement with the previous reports that the patterns of selected bacterial diseases have also been increased at a higher temperature.

The prevalence of *Pseudomonas sp*, *Aeromonas* sp and *Acinetobacter sp* has increased when the water temperature was around 29^{0}C. The increased rate of bacterial replication and transmission could be the reason for such enhanced prevalence in summer. The report from Ramos et al. (2013), explains that the prevalence of *Pseudomonas* increases during the summer irrespective of their species. Our study pertains to the reports of the high prevalence of *Pseudomonas* species during summer. The impact of environmental factors like temperature, humidity, and precipitation are a plausible explanation for such increment in *Pseudomonas* prevalence. However, we have also noted low dissolved oxygen (4.85 mg L^{-1}) concentration in particular reservoirs with high *Pseudomonas* abundance.

The most significant histopathological changes observed in gills, liver, kidney, and intestinal tissues in the fishes isolated with *Pseudomonas* and *Aeromonas sp* are suggestive of biotic stress in the reservoirs. Similar histopathological observations were reported by Ventura and Grizzle (1988); Candan (1990) due to bacterial infection in fishes. Kumar (2015) reported some important histological observations during the bacterial infection in the liver, kidney and gill tissues of pangas catfishes. Similar pathogens were also identified and isolated in the present study with the reported histological changes in the gill, kidney, and liver tissue.

The misuse of antibiotics has so long been discussed in aquaculture and many of them are banned in India (Aich et al. 2018). In the present study, isolated bacteria have shown resistance to Ampicillin (96%), Penicillin-G (80%), Sulphamethoxazole (32%), and Tetracycline (28%). While resistance to Ampicillin and Penicillin is intrinsic for most of the gram-negative bacteria (Munita and Arias, 2016; Exner et al. 2017), Tetracycline and Sulphamethoxazole are frequently used antibiotics for treating bacterial infection. Bacterial resistance to antibiotics reduces their application in disease control. Similarly, Sarter et al. (2007) reported multidrug resistance to Ampicillin, Oxytetracycline, and Sulphamethoxazole in bacteria isolated from the Pangas catfish. Bacteria isolated from other cage cultured fishes were also have shown drug resistance. Some of the resistant genes can also be horizontally transferred to other environmental microbes and human pathogenic bacteria which pose a risk for human health (Boran et al. 2013).

Conclusion

The present paper describes the prevalence of bacteria in the enclosure environment. Higher prevalence of opportunistic bacterial pathogens, in particular Pseudomonas and Aeromonas spp. indicates the presence of immense biotic stress to the fishes in cages. The disease susceptibility of fishes can be minimized by mitigating the stress factors in cages. For instance, optimum water and soil quality, ideal feeding, and stocking density and application of Immunostimulants can reduce disease loss in cages. The presence of zoonotic pathogens could also be a serious public health risk to fish consumers. Investigation and identification of specific risk factors will be helpful for spreading awareness regarding disease control and prevention strategies.

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