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Prevalence of *Aeromonas hydrophila* and *Pseudomonas fluorescens* and factors influencing them in different freshwater fish ponds

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Abstract

Real-time quantitative PCR (qPCR) was performed to elucidate the abundance of *Aeromonas hydrophila* and *Pseudomonas fluorescens*, which are among the most widespread fish pathogens in ponds. Both pathogens have three different breeding patterns, namely, (a) gibel carp (*Carassius auratus gibelio*), (b) yellow catfish (*Pelteobagrus fulvidraco*), and (c) black carp (*Mylopharyngodon piceus*), over a production season. Results revealed that pond sediments have significantly higher bacterial levels of *A. hydrophila* and *P. fluorescens* (10^5–10^6 copies μl^{-1} DNA) than pond water (10^3–10^4 copies μl^{-1} DNA). In addition, independent regression models revealed that environmental variables influence the levels of pathogenic bacteria. The occurrence of *A. hydrophila* and *P. fluorescens* were significantly positively correlated to dissolved oxygen and water temperature, respectively. On the contrary, both pathogens were negatively correlated to total nitrogen. In this study, the prevalence of pathogenic bacteria and their relationships with physicochemical factors in different pond environments were investigated for the first time through a molecular method. Furthermore, although we did not include fish diseases occurring during the production season, our results can provide useful theoretical information for fish breeding especially with regard to the prevention of related bacterial diseases.

Keywords: *Aeromonas hydrophila*, *Pseudomonas fluorescens*, Freshwater fish, qPCR, Ponds

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Introduction

Over the last 30 years, aquaculture industries have progressed in China, and capital-intensive production has become the main breeding pattern (Zhang *et al*., 2014). The total production increased from 1.23 million tons in 1979 to 45.41 million tons in 2013 because of the increase in intensive farming and total aquaculture area (Dong, 2014). However, bacterial diseases, which can cause substantial socio-economic losses, have increased and thus have become a major threat (Austin and Austin, 2012; Xu and Zhang, 2014). According to Austin and Austin (2012), bacterial diseases affecting freshwater fish are caused by *Vibrio*, *Salmonella*, *Aeromonas*, and *Pseudomonas*. *Aeromonas hydrophila* and *Pseudomonas fluorescens* are the most widespread fish pathogens. *A. hydrophila* is the causative agent of motile aeromonas septicemia (Harikrishnan *et al*., 2003) and infects several fish species, including tilapia (*Oreochromis* spp.), catfish (*Silurus asotus*), goldfish (*Carassius auratus*), common carp (*Cyprinus carpio*), and eel (*Anguilla japonica*; Pridgeon *et al*., 2011). By contrast, *P. fluorescens* is responsible for the hemorrhaging at the base fins (Austin and Austin, 2012). *A. hydrophila* and *P. fluorescens* are considered as opportunistic fish pathogens (Harikrishnan and Balasundaram, 2005; da Silva *et al*., 2012), and diseases caused by them are common in Chinese aquaculture ponds (Lu, 1992; Deng *et al*., 2011).

The outbreaks of fish diseases depend on several factors, such as quality of pond water, natural conditions, whether a fish is captured, and abundance of pathogenic bacteria (Ekwenye and Ugwoejf, 2009; Moore *et al*., 2014). Previously, using chemotherapeutic agents or antibiotics in aquaculture ponds to prevent different bacterial diseases in fish ponds was a common practice (Liu and Song, 2007). However, chemotherapeutic agents may cause stress to fish and pollute the environment when used in excessive amounts, while using antibiotics to modulate the "healthy gut microbiota" often produces antibiotic-resistant bacteria (Smith *et al*., 1994; Cabello, 2006; Ringø *et al*., 2014; 2016). Thus, early prevention is important. Furthermore, gaining knowledge on the abundance of important pathogens in ponds and their relationships with environmental factors is important as a precaution. However, to the best of our knowledge, no study has reported such research in China.

Real-time quantitative PCR (qPCR) is a rapid and sensitive method and is highly specific, and thus obtaining quantitative information through this method is easy in the presence of pathogens (Shannon *et al*., 2007; Liu *et al*., 2012). In the present study, we aimed to assess the abundance of *A. hydrophila* and *P. fluorescens* in different freshwater pond environments through qPCR and monitor the effects of environmental physicochemical factors. In addition, the present study is conducted to establish baseline information necessary for the formulation of pragmatic disease

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prevention and control, which have not been evaluated so far.

Materials and methods

Fish ponds with different breeding patterns

The present study was carried out in nine ponds with the following three traditional breeding patterns: gibel carp (pond MGC), yellow catfish (pond MYC), and black carp (pond MBC). Each breeding pattern included three rectangular replicate ponds with an average depth of 1.8 m. The ponds were randomly selected from two fish farms located in Jingzhou city, Hubei province, China. Water was mainly supplied by underground water. Different fish species and the number of fish cultured in the ponds are shown in Table 1.

Table 1: Management of different breeding ponds.

Management issue	Breeding patterns		
	MGC	**MYC**	**MBC**
Pond area	1×667 m^2	2.3×667 m^2	12×667 m^2
Stocking period	March–April	March–April	February–March
Stocking size (g/tail)	GC: 30.3, SC: 300, BC1: 150, BSB: 150	YC:7, SC:150, BC1: 450	BC: 750, GC:17, SC: 25, BC1: 150
Stocking density (Tail/667m^2)	GC: 2200, SC: 150, BC1:25, BSB: 50	YC:10000, SC: 50, BC1: 207	BC: 1200, GC: 300, SC: 250, BC1: 50

GC: gibel carp, YC: yellow catfish, BC: black carp, SC: silver carp (*Hypophthalmichthys molitrix*), BC1: bighead carp (*Aristichthys nobilis*), BSB: blunt snout bream (*Megalobrama amblycephala*), MGC: pond of mainly breeding gibel carp, MYC: pond of mainly breeding yellow catfish, MBC: pond of mainly breeding black carp.

Sample collection

Water and sediment samples were collected from the fishpond from the 20th to the 21st of each month from April to October 2013. This period is the production season. Water samples were collected using plastic containers (500 ml) approximately 30–50 cm from the water surface at three different locations (inlet, middle, and outlet) of each pond. Samples were pooled together as one representative sample to prevent spatial heterogeneity. Pooled water (250 ml) was first filtered through glass fiber filters (1.2 μm-large pores, Whatman type GF/C, England) and then through a cellulose acetate membrane (0.45-μm pore size, Millipore, USA). Both filters were collected and stored at −20 °C until total microbial DNA was extracted. The same amount of pooled water was used to assess chemical variables.

A Peterson dredge (PSC-1/16, Wuhan Yisai Co., LTD) was used to collect sediment samples from the middle location near the feeding platform of each pond. The method of collection was based on a previous study that evaluated optimum sampling points in ponds (Jing *et al*., 2009). All sediments were preserved in sealed bags at −20°C prior to microbial total DNA extraction. During the sampling period, no fish diseases were noticed, and the mortality rates of gibel carp in pond MGC, yellow catfish in pond MYC, and black carp in pond MBC had an average of 0.7%, 0.43%, and 0.06%, respectively, in each pond over the production season.

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No death was revealed for bighead carp and silver carp during the experiment.

Physicochemical parameters of pond water

Dissolved oxygen (DO), water temperature (T), and pH at the water surface were measured using a HACH-hq40d probe (HACH, USA). Total nitrogen (TN), total phosphorus (TP), phosphate (PO_4–P), nitrate nitrogen (NO_3–N), ammonia nitrogen (NH_4–N), and nitrite nitrogen (NO_2–N) were measured using a HACH DR2700 (HACH, Colorado, USA) according to the manufacturer's manual.

DNA extractions

The glass fiber filters and cellulose acetate membrane used to filter pooled water were cut into small pieces and soaked in 3 mL of sterile lysis solution (30 mmol of L^{-1} EDTA, 10 mmol L^{-1} of Tris-HCl, 05% sodium dodecyl sulphate (SDS), 0.1 mg of proteinase K, 0.05 mg of RNase A) overnight at 55 °C. This step was followed by standard phenol/chloroform extraction performed through a previously described method (Li *et al*., 2012a). DNA was precipitated with cold ethanol and pelleted by centrifugation at 13 000 g for 20 min at 4 °C. The pellets were washed with 70% ethanol and air-dried for 30 min and then resuspended in 50 µL of TE buffer solution. DNA preparation for the sediment samples (2.5 g for DNA extraction) was performed using an A.E.Z.N.A.TM Soil DNA Kit (OMEGA, USA) according to the manufacturer's instructions. All DNA solution was stored at −20°C until further use.

Quantifying A. hydrophila and P. fluorescens through qPCR

The absolute abundance of *A. hydrophila* and *P. fluorescens* were quantified through qPCR and by using standards constructed with known amounts of plasmid DNA. Briefly, the PCR products of 16S rRNA genes were gel-purified, and then cloned into pMD18-T vectors, and finally transformed into *Escherichia coli* cells. After confirmation by sequencing, plasmid DNAs-that contained the cloned 16S rRNA genes were extracted. DNA concentration was determined spectrophotometrically with a serial of 10-fold dilutions (from 1×10^4 to 1×10^8). Standard curves were then established using diluted plasmid DNA in qPCR. The abundances of *A. hydrophila*, *P. fluorescens*, and total bacteria in each water and sediment sample were then evaluated. Three repetitions were performed for each breeding pattern, and 18 samples (9 water samples and 9 sediment samples) were analyzed each month.

The qPCR analysis was carried out on an ABI 7500 FAST system (Applied Biosystems, Tacoma, Washington, USA) as described elsewhere (Li *et al*., 2013). Each PCRcontained 25 µl of 16SYBR Green qPCR master mix (Merck KGaA, Shanghai Ruian), 0.2 µmol ml^{-1} of each primer (Table 2), and 2 µL DNA templates (10 ng $µl^{-1}$). PCR cycling included an initial denaturation for 10 min at 95 °C, followed by 40 cycles of 95 °C for 10 s and 60 °C for 40 s.

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Fluorescence readings were collected at each extension step, and final melting analysis was performed to check nonspecific product formation. Each sample included three replicates.

Table 2: DNA sequences used for qPCR primers.

Assay	Primer code	Oligonucleotide sequence (5′–3′)	Reference
All bacteria	Eub338F	ACTCCTACGGGAGGCAGCAG	Fierer *et al*., 2005
	Eub518R	ATTACCGCGGCTGCTGG	
Aeromonas hydrophila	AER-F	GATTGCGGCCAACCAGTC	Meng *et al*., 2012
	AER-R	CCCCAGCGCAGGAAGC	
Pseudomonas fluorescens	XZP-F	GCCCGAAATTGGGTCTGTAG	Deng *et al*., 2010
	XZP-R	CCCCAAGCAATCTGGTTATACT	

Statistical analysis

For statistical purposes, the 16S rRNA gene copies for *A. hydrophila* and *P. fluorescens* in the culture were calculated and then $\log_{10}$ transformed. Environmental conditions that affected the abundance of pathogenic bacteria were investigated separately through independent regression models. Statistical analyses were performed using SPSS 18.0 (PASW statistics, USA), and the level of significance was set at a p value of <0.05.

Results

Abundance of A. hydrophila and P. fluorescens in different ponds

The abundance of *A. hydrophila* and *P. fluorescens* in different pond water and sediment samples were measured by qPCR from April to October. During the sampling period, copies μl^{-1} DNA of *A. hydrophila* in the water samples ranged from $2.10\times10^4\pm0.56\times10^4$ to $5.85\times10^5\pm1.57\times10^5$ in the MGC pond, from $1.17\times10^4\pm0.93\times10^4$ to $2.89\times10^5\pm2.65\times10^5$ in the MYC pond, and from $0.99\times10^4\pm0.72\times10^4$ to $5.42\times10^4\pm1.37\times10^4$ in the MBC pond. The abundance of *P. fluorescens* ranged from $0.86\times10^3\pm0.04\times10^3$ to $11.5\times10^3\pm7.27\times10^3$ in the MGC pond, from $0.71\times10^3\pm0.54\times10^3$ to $3.44\times10^3\pm0.91\times10^3$ in the MYC pond, and from $0.87\times10^3\pm0.28\times10^3$ to $2.31\times10^3\pm2.5\times10^3$ in the MBC pond (Table 3).

qPCR analysis on the sediment samples revealed that copies μl^{-1} DNA of *A. hydrophila* were from $1.04\times10^6\pm0.17\times10^6$ to $4.19\times10^6\pm0.51\times10^6$ in the MGC pond, from $1.15\times10^5\pm0.35\times10^5$ to $11.1\times10^5\pm9.99\times10^5$ in the MYC pond, and from $2.42\times10^5\pm3.04\times10^5$ to $7.54\times10^5\pm3.06\times10^5$ in the MBC pond. The abundance of *P. fluorescens* ranged from $0.19\times10^5\pm0.05\times10^5$ to $1.68\times10^5\pm0.42\times10^5$ in the MGC, from $0.19\times10^5\pm0.04\times10^5$ to $3.85\times10^5\pm0.69\times10^5$ in the MYC pond, and from $0.22\times10^5\pm0.06\times10^5$ to $5.40\times10^5\pm1.84\times10^5$ in the MBC pond (Table 4).

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Table 3: Quantification of the ribosomal gene copies of two pathogenic bacteria by qPCR from different pond water samples at different sampling times.

Sampling time	16S rRNA genes copy number of water sample (copies µl^{-1} DNA, mean±SD)					
	MGC		MYC		MBC	
	AER(×10^4)	PSE(×10^3)	AER (×10^4)	PSE (×10^3)	AER (×10^4)	PSE (×10^3)
Apr.	6.79 ± 2.34	3.98 ± 1.91	3.97 ± 1.52	2.2 ± 0.95	2.49 ± 0.4	1.51 ± 0.36
May	5.23 ± 4.49	4.09 ± 1.33	12.3 ± 8.9	3.21 ± 2.34	1.64 ± 2.09	2.31 ± 2.5
Jun.	2.10 ± 0.56	5.22 ± 4.94	2.9 ± 1.23	1.67 ± 0.24	1.01 ± 0.9	2.18 ± 1.85
Jul.	7.29 ± 2.09	11.5 ± 7.27	26.4 ± 11.2	3.44 ± 0.91	0.99 ± 0.72	1.73 ± 0.93
Aug.	9.44 ± 2.58	0.86 ± 0.04	14.1 ± 6.16	0.71 ± 0.54	5.42 ± 1.37	0.87 ± 0.28
Sep.	3.61 ± 0.83	2.43 ± 1.19	28.9 ± 26.5	1.50 ± 0.34	4.94 ± 4.03	2.30 ± 1.38
Oct.	58.5 ± 15.7	2.42 ± 0.36	1.17 ± 0.93	0.90 ± 1.10	2.02 ± 1.30	1.33 ± 0.88

MGC: pond of mainly breeding gibel carp, MYC: pond of mainly breeding yellow catfish, MBC: pond of mainly breeding black carp, AER: *Aeromonas hydrophila*, PSE: *Pseudomonas fluorescens*.

Table 4: Quantification of the ribosomal gene copies of the two pathogenic bacteria through qPCR from different culturing pond sediment at different sampling times.

Sampling time	16S rRNA genes copy number of sediment sample (copies µl^{-1} DNA, mean±SD)					
	MGC		MYC		MBC	
	AER(×10^6)	PSE(×10^5)	AER(×10^5)	PSE(×10^5)	AER (×10^5)	PSE(×10^5)
Apr.	1.21 ± 0.19	1.41 ± 0.71	6.65 ± 4.00	1.77 ± 0.14	3.00 ± 2.65	2.31 ± 0.45
May	1.79 ± 0.22	1.29 ± 0.25	7.41 ± 2.90	1.98 ± 0.45	3.55 ± 1.84	3.74 ± 2.96
Jun.	1.64 ± 0.23	1.68 ± 0.42	8.91 ± 4.75	2.43 ± 0.40	3.85 ± 2.73	4.98 ± 1.27
Jul.	1.04 ± 0.17	1.55 ± 0.36	1.15 ± 0.35	3.08 ± 0.36	2.42 ± 3.04	2.54 ± 0.62
Aug.	1.75 ± 0.35	1.67 ± 0.53	3.01 ± 1.73	3.85 ± 0.69	3.73 ± 2.86	3.81 ± 1.46
Sep.	2.57 ± 0.28	1.65 ± 0.63	11.1 ± 9.99	3.40 ± 0.35	5.74 ± 1.19	5.40 ± 1.84
Oct.	4.19 ± 0.51	0.19 ± 0.05	9.09 ± 7.08	0.19 ± 0.04	7.54 ± 3.06	0.22 ± 0.06

MGC: pond of mainly breeding gibel carp, MYC: pond of mainly breeding yellow catfish, MBC: pond of mainly breeding black carp, AER: *Aeromonas hydrophila*, PSE: *Pseudomonas fluorescens.*

Temporal and spatial changes of A. hydrophila and P. fluorescens in ponds

The ratio of *A. hydrophila* and *P. fluorescens* to all bacteria was calculated and transformed to $\log_{10}$ to compare the temporal and spatial changes of the pathogenic bacteria in different ponds. The general trends of *A. hydrophila* population in the MGC and those of the MYC ponds were similar but with large fluctuations during sampling. However, the relative abundances of the pathogens in the MBC pond varied. In particular, the abundance decreased initially, and then increased, and finally decreased again (Fig. 1a). The temporal changes in *A. hydrophila* in the pond sediments were evident in different breeding patterns (Fig. 1b). In the pond water and sediment samples (Fig. 1), the abundance of *A. hydrophila* in the MGC and that of the MYC ponds were significantly higher (p<0.05) than that in the MBC pond.

Temporal changes observed in *P. fluorescens* in the pond water revealed a similar trend, as indicated by the qPCR results. In particular, a decrease of *P. fluorescens* abundance was observed from April to October (Fig. 1c). In the pond sediments of MYC and MBC, the abundance of *P. fluorescens* had minimal change during the sampling periods, whereas the abundance of *P. fluorescens* in the MGC pond was significantly (p<0.05) lower than those of the other ponds (Fig. 1d).

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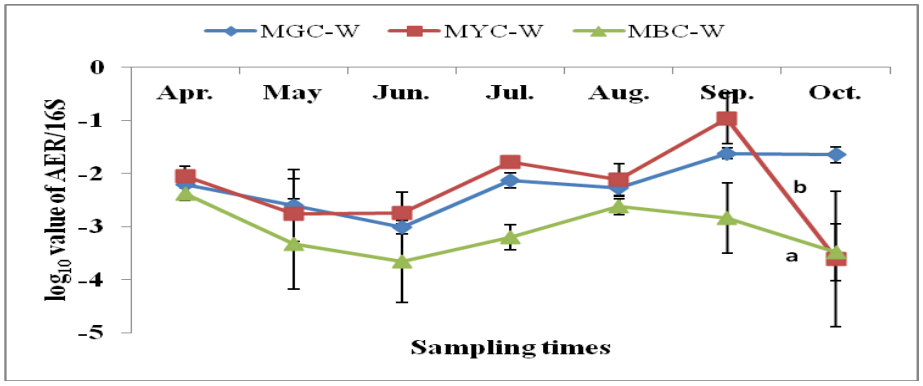
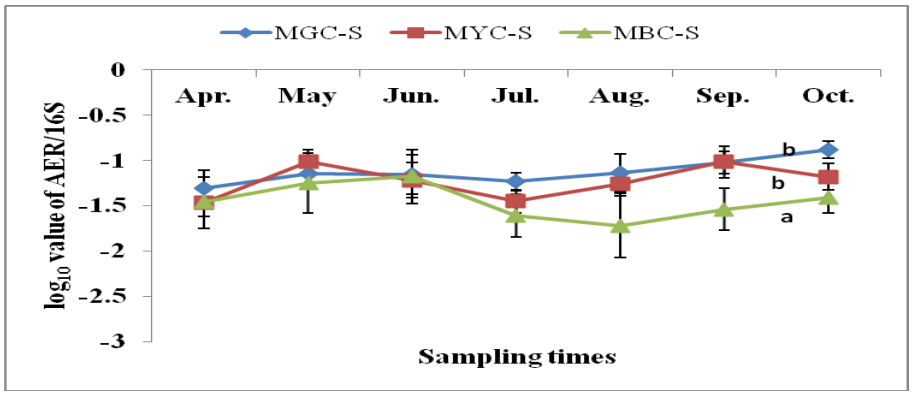


a



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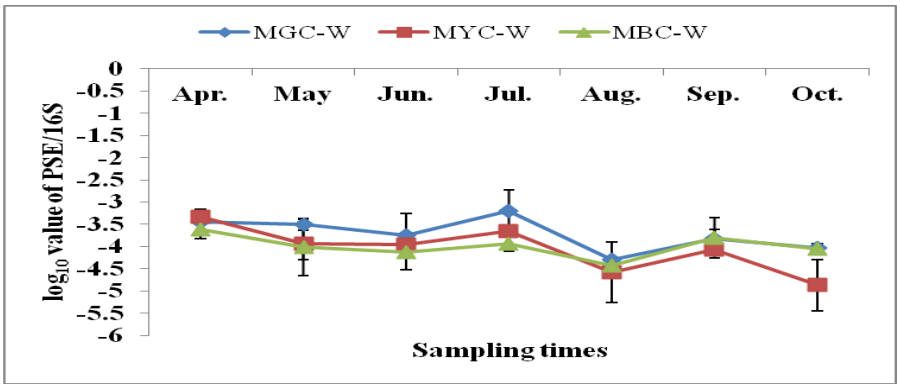

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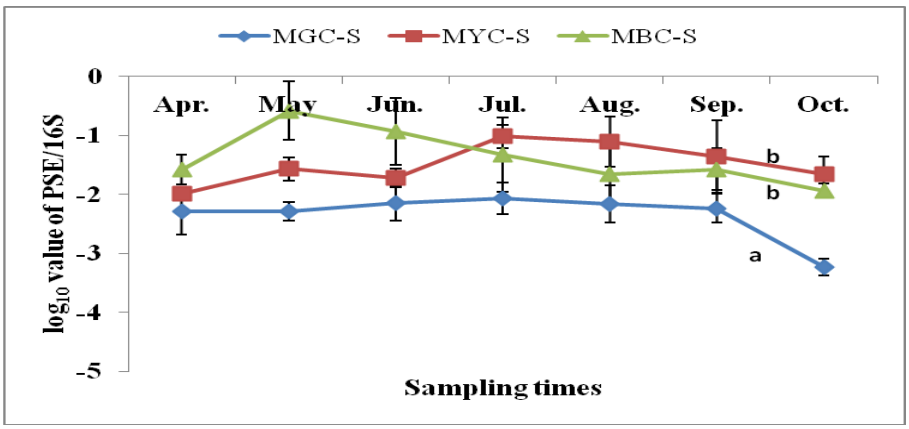

d

Figure 1: Temporal changes observed in *Aeromonas hydrophila* (a [water] and b [sediment]) and *Pseudomonas fluorescens* (c [water] and d [sediment]). The results are based on the relative abundances determined by qPCR. MGC: pond of mainly breeding gibel carp, MYC: pond of mainly breeding yellow catfish, MBC: pond of mainly breeding black carp. W: water; S: sediment. a,b represent significant (p<0.05) difference.

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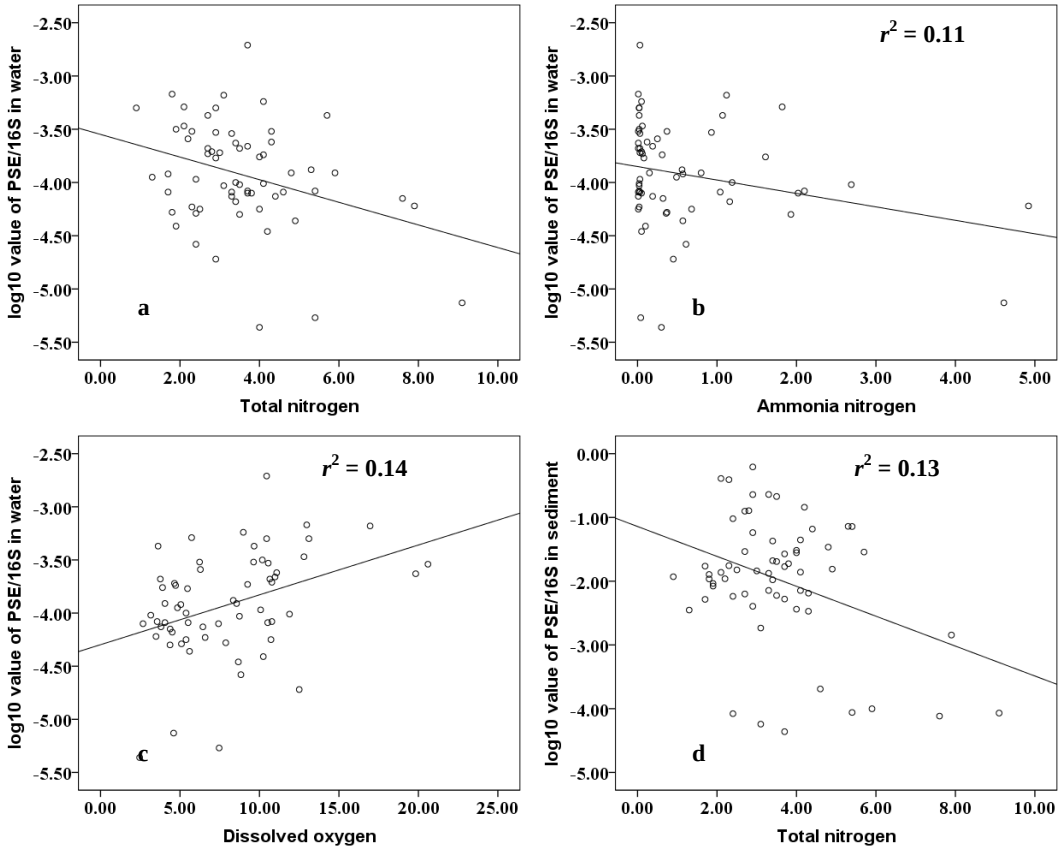
Influence of environmental factors on A. hydrophila and P. fluorescens

Environmental variables that influenced the abundance of *A. hydrophila* and *P. fluorescens* were identified. Fig. 2 shows the statistical associations between the variables and relative abundances of the two pathogenic bacteria. In the pond water samples, TN and NH_4–H negatively influenced the abundance of *P. fluorescens*, whereas DO had a positive effect, as indicated by the results from independent regression analysis (Fig. 2a, 2b, 2c, r^2=0.11, 0.06, and 0.14, respectively). In the sediment samples, TN had significantly negative relationship with *P. fluorescens* abundance (Fig. 2d, r^2=0.13), while water temperature had a positive influence (Fig. 2e, r^2=0.31).

TN, which had a significant effect, was the only factor related to *A. hydrophila* abundance in the pond water samples (Fig. 2f, r^2=0.12). In comparison, the results revealed that phosphate and TP were significant factors that positively influenced *A. hydrophila* abundance in the pond sediments. In Fig. 2h, g, r^2=0.07 and 0.08, respectively.



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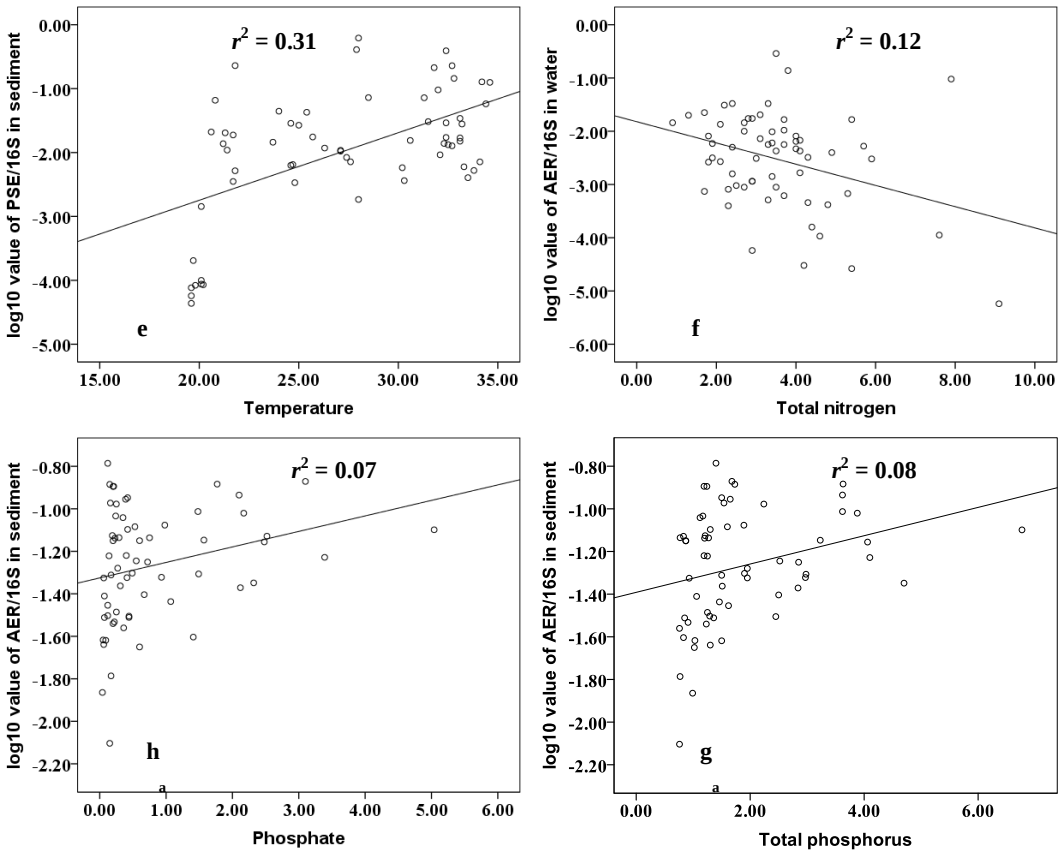

Figure 2: Predicted probabilities for the detection of relative density of the two pathogenic bacteria related to environmental factors. (a)–(c) for *Pseudomonas fluorescens* in pond water and (d)–(e) *for Pseudomonas fluorescens* in pond sediment, (f) for *Aeromonas hydrophila* in pond water, and (h)–(g) *for Aeromonas hydrophila* in pond sediment.

Discussion

A. hydrophila and *P. fluorescens* are opportunistic fish pathogens and widely distributed in freshwater fishponds (Nielson *et al*., 2001; Akinbowale *et al*., 2007). In the present study, the abundance of *A. hydrophila* and that of *P. fluorescens* in three freshwater fishponds with three breeding models, mainly gibel carp (MGC), yellow catfish (MYC), and black carp (MBC) were determined. These fish species are commonly used in pond aquaculture in the Hube Province, China (Zhang and Tan, 1989; Ma, 2011).

The abundances of *A. hydrophila* (~10^4) and *P. fluorescens* (~10^3) in pond waters were lower than those in the pond sediments (~10^5–10^6). The high bacterial loads in the pond sediments were in agreement with the previous findings of Al-harbi and Uddin (2004, 2005), while the bacterial counts range from 10^6 cfu g^{-1} to 10^8 cfu g^{-1} in the sediments of brackish water ponds in Saudi Arabia. Zhang *et al*. (2008) and Li *et al*. (2012b) documented that *Vibrio* and *Aeromonas* are prevalent in pond sediments in China. The increase in bacterial loads in the sediments may be attributed to the abundance of organic matters, which are nutrient sources used by various bacteria (Al-harbi and Uddin, 2005). The fish samples appeared to be healthy during the sampling, and no fish

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morbidity caused by the two pathogenic bacteria was documented, This result suggests that the abundances of *A. hydrophila* and *P. fluorescens* had $<10^4$ copies μl^{-1} DNA and $< 10^3$ copies μl^{-1} DNA, respectively in the pond water samples, and both bacteria had $<10^5$ copies μl^{-1} DNA in the pond sediment samples. Furthermore, both bacteria can be tolerated by gibel carp, yellow catfish, and black carp. Although this hypothesis merits further investigations, our data may serve as baseline information for fish farmers engaged in gibel carp, yellow catfish, and black carp culture in freshwater ponds in China. However, the zoonotic potential of the two pathogenic bacteria evaluated in the present study should not be disregarded, as pond technicians are constantly exposed or in contact with fish and the environment (Pakingking *et al*., 2015).

The dynamic changes observed in the two pathogenic bacteria in the three ponds were different. Furthermore, the abundance of *A. hydrophila* in MBC was lower compared to those in the other ponds. The large surface area of MBC that enables self-healing and maintain the water quality may explain the lower pathogenic abundance as previously documented (Juszczak *et al*., 2007).

Understanding the relationships between environmental factors and pathogenic bacteria are of importance to regulate the cultured water quality. In the current study, TN and NH_4–N had significant negative effects on *A. hydrophila* and *P. fluorescens* in pond water, indicating that the presence of the pathogens may decrease at increased TN and NH_4–H concentrations. This result is in disagreement with those of a previous study, which reported that high NH_4–N concentration in aquaculture water can cause bacterial disease or even death of cultured freshwater fish (Wang *et al*., 2013). The abundance of *P. fluorescens* increased with DO concentrations in the pond water, possibly because the bacterium is strictly aerobic and thus requires oxygen to perform respiratory metabolism (Austin and Austin, 2012). Meanwhile, water temperature had significant positive correlations to *P. fluorescens* abundance, suggesting that reducing water temperature can decrease the abundance of *P. fluorescens*. This result is in accordance with that of Olanya *et al*. (2014), who reported that water temperature is an important factor that regulates *P. fluorescens* abundance. Meanwhile, *A. hydrophila* abundance in pond sediment, reducing phosphate and TP concentrations may control the density of the bacterium in pond sediments, because *A. hydrophila* can produce phosphatase. Furthermore, pond sediments are rich in phosphate and TP (Zhang *et al*., 2014).

Regular monitoring of pathogenic bacteria and physicochemical factors in the water and sediments of freshwater fishponds through molecular methods, such as qPCR, monthly or weekly can be instituted. Wu *et al*. (1994) documented the importance of preventing fish injury to reduce the invasion of pathogenic bacteria. In addition, biological elements must also

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be considered in practical culture, especially of fish. Pakingking *et al*. (2015) reported that pond water and sediment bacteria directly influence the bacterial microbiota present in the gills and intestines of several fish species.

In conclusion, *A. hydrophila* and *P. fluorescens* are more prevalent in freshwater fish pond sediments than those in the studied pond water. The abundance of *A. hydrophila* and *P. fluorescens* were $<10^4$ and $<10^3$ copies μl^{-1} DNA, respectively, in pond water, and the abundances of both bacteria were $<10^5$ copies μl^{-1} DNA in the pond sediment. Furthermore, both bacteria can be tolerated by gibel carp, yellow catfish, and black carp, which are commonly cultured in China. The occurrences of *A. hydrophila* and *P. fluorescens* were positively and significantly correlated to DO concentration and water temperature, respectively. However, both occurrences were negatively correlated to TN concentration. In future studies, the influence of these environmental factors on pathogenic bacteria will be investigated.

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