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Assessment of Bacteria and Water Quality Parameters in Cage Cultured *Pangasius hypophthalmus* in Temerloh, Pahang River, Malaysia

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ABSTRACT

Pangasius hypophthalmus or famously known by local Malaysians as Patin Hitam is one of the most important sources of food in Malaysia. It is widely cultured in the Peninsular Malaysia especially in Pahang as Patin and is a popularly consumed freshwater fish. Global economic interest in the fish has increased its demand in the USA and Europe. However, high mortality due to bacterial and viral infections is the main problem that needs to be solved. Therefore, bacteria in *P. hypophthalmus* in Pahang is being focused with the factors connected to the prevalence of bacteria and virus in *P. hypophthalmus*. This research was conducted for two cycles (February-September 2016 and January-August 2017) in different farms in Temerloh, Pahang. Bacteria and virus samples were taken from three organs of Patin Hitam which are kidney, liver and spleen. Physical parameters for water quality were measured using a multi-parameter probe sensor (YSI, USA) and chemical parameters were analysed with DR900 colorimeter (Hach, USA). Bacteria samples were identified using biochemical test kits, API 20NE and 20E, followed by confirmation of the bacteria using Polymerase Chain Reaction (PCR). Virus samples were identified using conventional PCR. There are several bacteria isolated throughout the culture period. The highest prevalence of *Aeromonas hydrophila* in Temerloh in the first cycle was in May 2016 (40%), however, was equally evident in four out of the seven months of the second cycle, which was in April, May, June and July 2017 (20%). There was a relationship between the prevalence of *A. hydrophila* and iron, nitrite and pH in the first cycle in Temerloh. However, there was no relationship in the second cycle. Significantly, these results could contribute to better treatment of fish disease and development of standard operating procedure of future fish culture for early disease prevention.

INTRODUCTION

Fish farming is one of the most important sectors that contribute economically in Malaysia. In Malaysia, catfish is the largest freshwater aquaculture species being cultured including, *Pangasius* spp., *Clarias* spp. and *Mystus numerus*. *Pangasius* spp. is one of the largest and most important freshwater fisheries in the world and Vietnam producing more than 1.1 million tons in 2008. Other producers are Thailand, Cambodia, Laos People's Democratic Republic, Myanmar, Malaysia, Bangladesh and China (FAO 2009). The production of *Pangasius* spp. in Malaysia showed tenfold increase from 1,625.21 tons in 2000 to 10,891.51 tons in 2011. Although the production of *Pangasius* spp. is increasing, it is reported to face disease problem causing 30% mortality especially in Sungai Pahang due to multiple combination of bacterial and viral infections. It has been reported that Patin Hitam or *Pangasuis* spp. were susceptible to diseases such as aeromonad infection or Motile Aeromonas Septicemia (MAS) caused by *Aeromonas* spp. (Subagja et al. 1999, Ferguson et al. 2001), bacillary necrosis of Pangasius (BNP) caused by *Edwardsiella ictaluri* (Crumlish et al. 2002, Asnor et al. 2018), Channel Catfish Virus caused by herpes virus (Siti Zahrah et al. 2013), and some ecto- and endoparasites infestations (Mavuti et al. 2012, Amir et al. 2018).

MAS is caused by any of the three species of the genus *Aeromonas* which are *Aeromonas hydrophila*, *A. caviae* and *A. sobria*. These species are commonly referred to as motile aeromonads (Hanson et al. 2014). The widespread distribution of these bacteria in the aquatic environment and the stress induced by intensive culture practices predisposes fish to infections. Involvement of bacteria is very vital in producing diseases in the farmed fishes in Bangladesh (Chowdhury 1998, Bikram et al. 2019). Channel catfish virus (CCV) disease is an acute infection of cultured fry and fingerling channel catfish (*Ictalurus punctatus*) which occurs mainly during summer and is often in fish less than four months old but with a few exceptions (Plumb 1986, Jing et al. 2018).

The environmental conditions which include availability of iron, oxygen levels, osmotic strength, pH, rainfall, water quality and temperature, and poor management practices (inadequate nutrition, overcrowding and overfeeding) can cause stress to the cultured fish and thus, making them more vulnerable to disease outbreaks (Winton 2001, Feng 2018). However, the information on the effect of water quality on the presence and susceptibility of fish to MAS and CCV infection diseases under the field conditions are limited. In this paper, the prevalence of bacteria and virus and factors associated with the infections were studied.

MATERIALS AND METHODS

Sample collection and bacterial isolation from fish, water and sediment: Ten fish from each farm were collected and dissected on field. Bacteria from kidney, liver and spleen tissues were swabbed and streaked onto Tryptic Soy Agar (TSA) media plates. Samples were brought back to the laboratory and were incubated at 30°C for 18 h (Pridgeon 2011, Khairunnisa et al. 2018). Single colonies were isolated to obtain pure colony cultures. Bacteria from water and sediment samples collected were also isolated to obtain pure colony.

Bacterial identification (API and PCR kit): From pure culture of bacteria, gram staining was conducted to classify the bacteria as gram positive or gram-negative bacteria. Next was biochemical tests for catalase and oxidase enzymes. After the classification, gram positive bacteria were identified using API 20E kit, while gram negative bacteria were identified using API 20NE kit. Bacteria were further identified by 16S rRNA PCR using universal primer 27F/1492R with the following amplification protocol: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30s (denaturation), 55°C for 30 s (annealing) and 72°C for 30s (extension), and a final extension of 72°C for 10 min (Wu 2012). The amplified PCR products were then sent for sequencing (First Base Laboratories, Serdang, Malaysia). Sequence data obtained were analyzed using the basic local alignment search tool (BLAST) available at the National Center for Biotechnology Information (NCBI) (http//:www.ncbi.nlm.nih.gov/BLAST) to obtain bacterial species identity defined by 97% homology.

Water quality observation (physical and chemical): For physical parameters of water, the data of temperature, pH and dissolved oxygen were recorded using multi-parameter probe sensor (YSI, USA). Water samples were brought to the laboratory for water chemical content observation which include nitrite, sulfide, ammonia and iron measured by DR900 colorimeter (Hach, USA).

Statistical analysis: Data were analyzed by a one-way ANOVA with the assumptions of normal distribution and homogeneity of variance were checked by the Shapiro-Wilk and the Levene's tests, respectively. Tukey's post-hoc test was used for comparisons between means with significant differences ($p < 5$). The statistical analyses were run with IBM SPSS v.20 software for Windows.

RESULTS AND DISCUSSION

Bacteria distribution: There were several bacteria identified throughout the first and second cycle of observation. Common bacteria found were *A. hydrophila, Photobacterium damselae, Pseudomonas fluorescens, P. luteola* and *Plesio-*

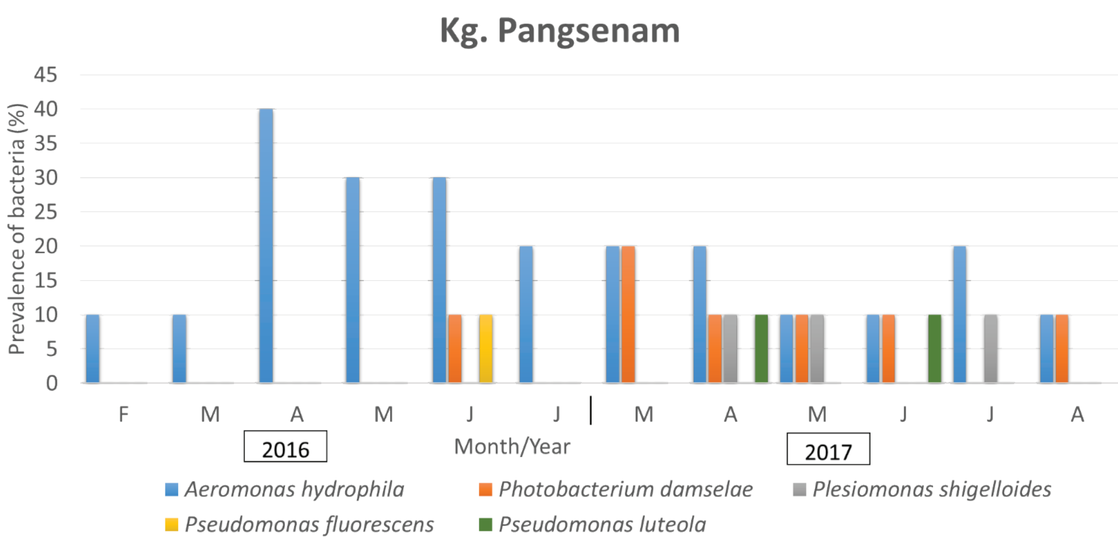

Fig. 1: The distribution of bacteria in Farm 1, Temerloh, Pahang.

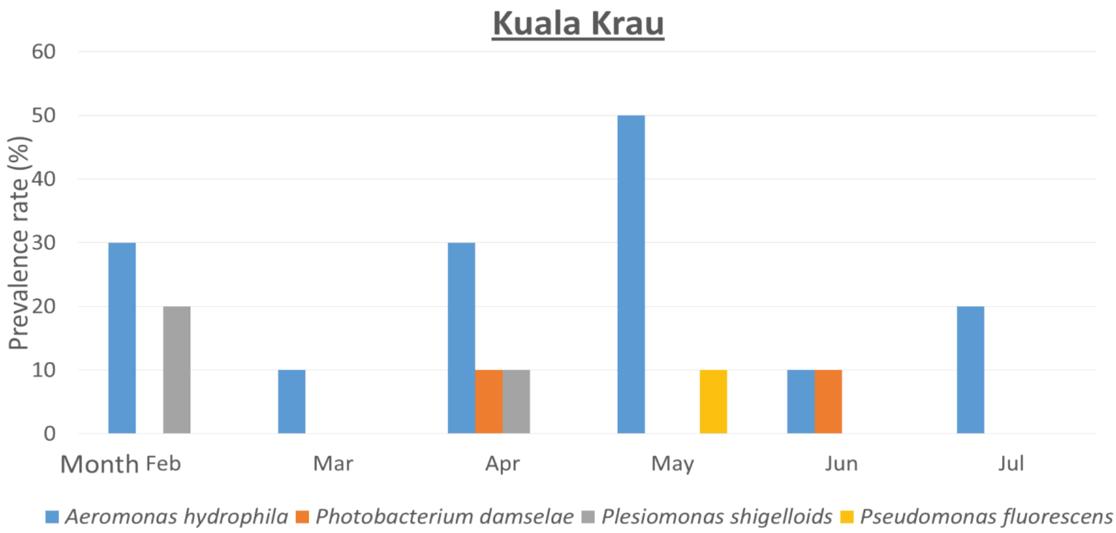

Fig. 2: The distribution of bacteria in Farm 2 (2016), Temerloh, Pahang.

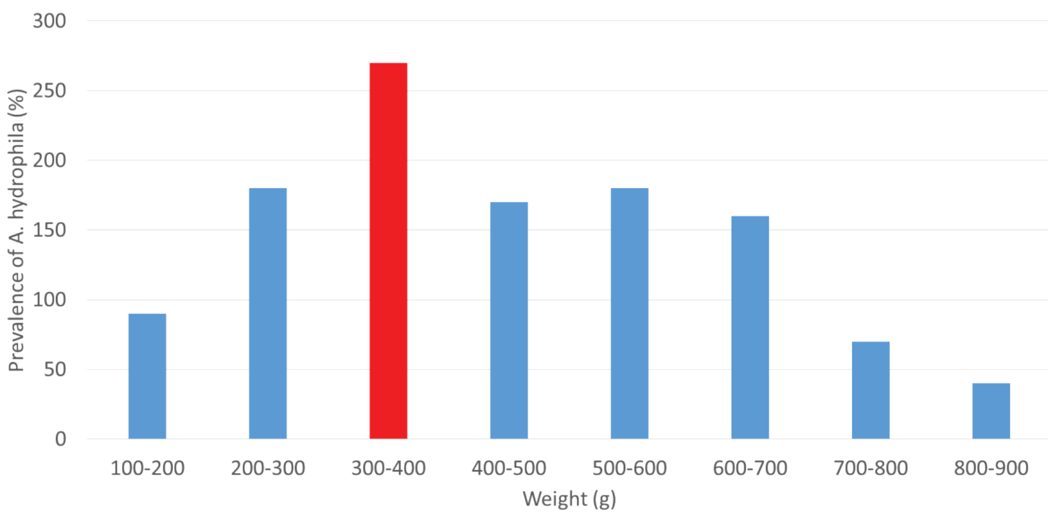

Fig. 3: The distribution of bacteria in Farm 2 (2017), Temerloh, Pahang.

monasshigelloides. From the observed results in both farms, *A. hydrophila* dominated the distribution of bacteria which was 63%, followed by *P. damselae* (23%), *P. shigelloides* (7%), *P. luteola* (4%) and *P. fluorescens* (3%). These results support the findings by Siti Zahrah (2013) which stated that *Pangasius* spp. is susceptible to *A. hydrophila* that causes MAS. This includes septicaemia, abdominal dropsy or ascites and evidence of blood-tinged peritoneal fluid which are typical signs of *A. hydrophila* infections in warm-water fish species (Monaem et al. 2018, Roberts 1993). From the gross clinical observation of infected fish, small lesions, abdominal distension, yellow ascites, and local haemorrhages in gills, fins and mouth were observed. *A. hydrophila* is the etiological agent of MAS which causes disease in a wide range of freshwater fish species (Sunny 2018, Newman 1993) and is frequently associated with stressed or immunocompromised fish (Roberts 1993).

There were differences in the presence of bacteria between two cycles of sampling. In the first cycle, there was no *P. luteola* isolated and *P. fluorescens* was present throughout the cycle. Meanwhile, in the second cycle sampling of both farms, *P. luteola* managed to be isolated and *P. fluorescens*

Table 1: Mean rate±SD of physicochemical water quality in different months at Farm 1, 2016.

Kg. Pangsenam (2016)						
	Feb	Mar	Apr	May	Jun	July
T (˚C)	28.83±0.12[d]	30.10±0.10[c]	32.07±0.06[a]	30.27±0.25[bc]	30.60±0.10[b]	28.37±0.12[e]
DO	10.13±0.3[a]	9.23±0.74[a]	7.67±0.25[b]	5.93±0.15[c]	5.60±0.44[c]	5.80±0.10[c]
pH	8.47±0.21[ab]	9.23±0.74[a]	7.67±0.25[b]	5.93±0.15[c]	5.60±0.44[c]	5.80±0.10[c]
$N0_2$	0.04±0.02[ab]	0.02±0.01[ab]	0.01±0.03[b]	0.02±0.01[ab]	0.04±0.01[a]	0.03±0.01[ab]
NH_3	0.17±0.02[ab]	0.13±0.06[bc]	0.05±0.02[c]	0.14±0.02[bc]	0.15±0.02[b]	0.25±0.05[a]
Fe	1.26±0.06[ab]	1.24±0.02[bc]	1.40±0.10[c]	1.44±0.05[bc]	1.30±0.11[bc]	1.32±0.10[a]
SO_2	2.53±0.15[e]	6.60±0.10[d]	7.00±0.10[cd]	9.00±0.10[b]	12.1±0.10[a]	8.67±1.52[bc]

[a,b,c,d,e] Different superscripts indicate values with significant difference (p<0.05).

Table 2: Mean rate±SD of physicochemical water quality in different months at Farm 1, 2017.

Kg. Pangsenam (2017)						
	Mar	April	May	Jun	July	Aug
T (˚C)	30.23±0.15[a]	27.93±0.15[b]	27.63±0.15[b]	27.63±0.15[b]	27.87±0.12[b]	27.8±0.24[b]
DO	5.95±0.07[a]	5.94±0.24[a]	5.80±0.04[a]	6.22±0.3[a]	5.91±0.1[a]	5.85±0.1[a]
pH	8.25±0.36[a]	6.80±0.29[b]	8.00±0.08[a]	8.03±0.13[a]	6.79±0.28[b]	7.9±0.14[a]
NO_2	2.33±0.03[a]	1.67±0.02[a]	5.33±0.04[a]	2.67±0.03[a]	5.00±0.03[a]	4.00±0.03[a]
NH_3	0.15±0.04[a]	0.27±0.08[a]	0.21±0.08[a]	0.20±0.05a	0.20±0.02[a]	0.17±0.01[a]
Fe	0.67±0.24[b]	1.38±0.19[a]	0.97±0.21[ab]	1.30±0.16[a]	1.15±0.13[ab]	1.10±0.11[ab]
SO_2	0.07±0.03[a]	0.07±0.02[a]	0.07±0.02[a]	0.11±0.03[a]	0.08±0.02[a]	0.10±0.02[a]

[a,b,c,d,e] Different superscripts indicate values with significant difference (p < 0.05).

Table 3: Mean rate±SD of physicochemical water quality in different months at Farm 2, 2016.

Kuala Krau (2016)						
	Feb	Mar	Apr	May	Jun	July
T (˚C)	27.83±0.85[c]	30.2±0.10[b]	32.87±0.21[a]	30.33±0.31[b]	29.97±0.15[b]	28.6±0.10[c]
DO	10.43±0.78[a]	8.63±0.38[b]	7.7±0.10[b]	5.83±0.15[c]	5.77±0.31[c]	6.06±0.32[c]
pH	8.53±0.32[a]	7.4±0.2[b]	7.13±0.21[b]	6.93±0.32[b]	7.33±0.25[b]	7.53±0.25[b]
NO_2	3.33±0.03[a]	4.33±0.23[a]	1.67±0.15[a]	3.33±0.21[a]	5.33±0.03[a]	1.67±0.05[a]
NH_3	0.10±0.03[a]	0.07±0.02[a]	0.12±0.03[a]	0.11±0.02[a]	0.06±0.05[a]	0.14±0.03[a]
Fe	0.27±0.03[b]	0.27±0.10[a]	0.40±0.06[a]	0.34±0.15[a]	0.25±0.03[a]	0.23±0.11[a]
SO_2	3.67±1.53[a]	6.33±7.57[a]	5.00±1.00[a]	12.33±4.93[a]	6.33±2.09[a]	6.33±3.79[a]

[a,b,c,d,e] Different superscripts indicate values with significant difference (p<0.05).

was absent. This might be because of the temperature of water that was higher in the first cycle than in the second cycle. *P. fluorescens* is present when temperature is elevated, meanwhile *P. luteola* exists in the damp and moist environment (Figs. 1-3).

Water quality observation: Water quality affects the health of fish and organisms that are in the aquatic environment. Poor water conditions can cause disease as a reflection of the interactions between the host fish and the disease-causing situation or stressors (Winton 2001, Tracy et al. 2018). In this paper, there were several parameters observed as disease causing situation which were later identified its correlation with diseases. The monthly mean and standard deviation of physical and chemical water quality in Temerloh, Pahang River for the two period cycles (2016-2017) were recorded in Tables 1-4.

Based on Table 1, temperature increased from February

Table 4: Mean rate±SD of physicochemical water quality in different months at Farm 2, 2017.

Kg. Bintang (2017)						
	March	April	May	Jun	July	Aug
Temp	31.78 ± 0.10^{a}	30.71 ± 0.10^{a}	30.60 ± 0.10^{a}	30.52 ± 0.10^{a}	30.4 ± 0.10^{a}	30.220 ± 0.10^{a}
DO	6.49 ± 0.08^{a}	6.77 ± 0.08^{a}	6.90 ± 0.08^{a}	7.11 ± 0.08^{a}	7.12 ± 0.08^{a}	7.25 ± 0.08^{a}
pH	7.99 ± 0.05^{a}	7.65 ± 0.05^{a}	7.76 ± 0.05^{a}	7.58 ± 0.05^{a}	7.55 ± 0.05^{a}	7.43 ± 0.05^{a}
$N0_2$	4.33 ± 0.03^{b}	0.03 ± 0.01^{ab}	0.11 ± 0.03^{ab}	0.03 ± 0.01^{ab}	0.04 ± 0.01^{a}	0.03 ± 0.01^{a}
NH_3	0.14 ± 0.03^{b}	0.22 ± 0.04^{a}	0.17 ± 0.03^{ab}	0.18 ± 0.02^{ab}	0.16 ± 0.03^{ab}	0.17 ± 0.02^{ab}
Fe	0.59 ± 0.5^{b}	1.87 ± 0.14^{a}	1.93 ± 0.11^{a}	1.82 ± 0.17^{a}	1.83 ± 0.06^{a}	1.89 ± 0.12^{a}
SO_2	0.13 ± 0.02^{a}	0.17 ± 0.03^{a}	0.16 ± 0.02^{a}	0.14 ± 0.02^{a}	0.16 ± 0.02^{a}	0.13 ± 0.02^{a}

[a,b,c,d,e] Different superscripts indicate values with significant difference ($p < 0.05$).

to April but slowly decreased from May to July. The highest temperature was in April 2016. There were no significant differences between the mean of temperature in March, May and June. However, the mean temperature in February, April and July showed significant differences where $p<0.05$. There were no significant differences for dissolved oxygen (DO), pH, nitrite, ammonia, iron and sulphide from February to July 2016 in Farm 1.

Temperature showed decrement at Farm 1 from March to August 2017 and was overall much lower compared to the temperature of water during 2016 at the same farm. Other physicochemical water showed no significant differences ($p > 0.05$) each month.

At a different site in 2016, the temperature of water recorded had a similar trend to Farm 1 in Temerloh where it was increasing until April but started to decrease until July 2016. Other parameters showed no significant differences accordingly. In Table 4, temperature decreases subsequently from March to August 2017 at Farm 2. Other water quality parameters also did not show any significant differences throughout the cycle.

The relationship between prevalence of *A. hydrophila* with physicochemical water quality: Fish diseases such as epidermal papilloma (Ottesen et al. 2007), gill hyperplasia (Cengiz 2006, Wali et al. 2018), neoplasia (Shiwanand et al. 2013), ulceration (Faruk 2008), and parasitic or viral infections (Molnár et al. 2006) were reported to be associated with

Table 5: The relationship between prevalence of *A. hydrophila* and physicochemical water quality at Farm 1 (2016-2017).

Sampling Sites	Parameters	Pearson's Correlation	P Value
Kg. Pangsenam (2016)	Iron	*0.8880	*0.0181
	NH_3	-0.3570	0.4873
	NO_2	-0.8528	0.0309
	pH	-0.8510	0.0316
	Sulphide	0.5381	0.2707
	Temperature	0.7834	0.0653
	DO	-0.5974	0.2105
Kg. Pangsenam (2017)	Iron	0.122	0.818
	NH_3	0.094	0.859
	NO_2	0.281	0.590
	pH	0.455	0.365
	Sulphide	0.295	0.570
	Temperature	-0.421	0.406
	TSS	-0.271	0.604
	DO	0.344	0.504

Table 6: The relationship between prevalence of *A. hydrophila* and physicochemical water quality at Farm 2 (2016-2017).

Sampling Sites	Parameters	Pearson's Correlation	P Value
Kaula Krau	Iron	0.237	0.608
	NH_3	0.208	0.654
	NO_2	0.255	0.255
	pH	-0.383	0.396
	Sulphide	-0.501	0.252
	Temperature	-0.059	0.900
	TSS	0.232	0.616
	DO	-0.269	0.560
Kg. Bintang	Iron	0.320	0.484
	NH_3	0.481	0.274
	NO_2	0.606	0.149
	pH	-0.722	0.065
	Sulphide	0.689	0.087
	Temperature	-0.140	0.765
	TSS	0.607	0.148
	DO	0.510	0.242

the aquatic environment conditions or pollution. The cause for these diseases has often been linked to contaminated diets (Sherif et al. 2013) or pollutants such as nitrogenous compounds, ammonia (Benli et al. 2008) and nitrites (Svobodova et al. 2005, Xiao et al. 2018), hydrocarbons (Noreña-Barroso et al. 2004), and heavy metals (Abedi et al. 2012) which can come from pesticides (Murthy et al. 2013, Zaheer et al. 2018) and sewage (Maceda-Veiga et al. 2013). The pollutants may weaken the fish metabolism and immune system and thus, leading to the colonization of microorganism and the development of clinical disease.

Based on Table 5, the relationship between water quality and the presence of *A. hydrophila* was observed. The results showed that there were relationships between iron, nitrite and

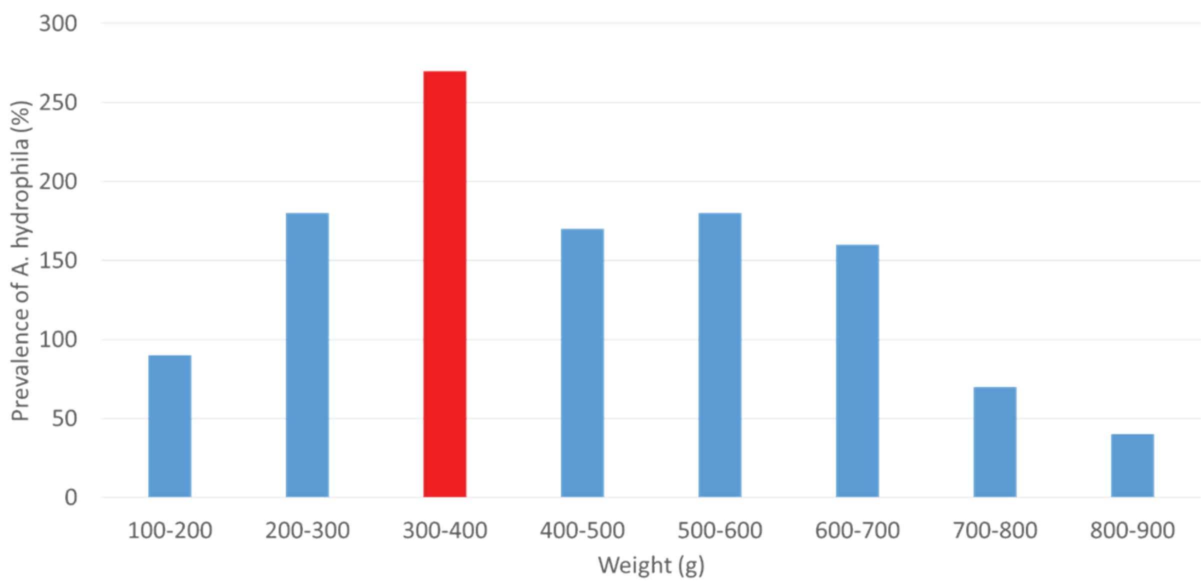

Fig. 4: Weight and prevalence of *A. hydrophila*.

pH in the first cycle of observation at Farm 1 in 2016 where $p < 0.05$. Meanwhile, in the second cycle at the same farm, no correlation was observed. This might be because of the sand mining activity that occurred during the monitoring period in 2016. On the other hand, there was no correlation between the physicochemical parameters of water quality and the presence of *A. hydrophila* at Farm 2 in 2016 and 2017.

Weight and prevalence of *A. hydrophila*: Isolations of *A. hydrophila* were significantly higher from *P. hypophthalmus* that weighed between 300-400g (Fig. 4). During this size, Patin Hitam are in the grow-out phase and their cage became overcrowded. Factors of high-water temperature, suboptimal oxygen level and overcrowding combined, made diseases to be easily transmitted from fish to fish or from environment to the fish. This result support a statement from Rasmussen-Ivey (2016) which asserted that disease outbreaks occur when the fish are in stressful condition.

CONCLUSION

In conclusion, there are several bacteria isolated which are *A. hydrophila, P damselae, P. fluorescens, P. shigelliodes* and *P. luteola.* There were different isolation patterns between 2016 and 2017. As for physicochemical water quality, correlation was observed only at Farm 1 in 2016 between iron, nitrite, pH and the presence of *A. hydrophila* where $p < 0.05$.

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