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Project Title: STUDY ON THE CONTAMINATION OF NAJS MUTAWASSITAH IN FISH AND WATER OF THE KUANTAN RIVER

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Abstract

Halal food should be free from najs (filth). The Kuantan River is very important in term of recreation, ecology and fish supply. It has a wide variety of fishes, which is regularly marketed for human consumption. It receives a lot of untreated sewage waste, which may carry enormous level of enteric bacteria. Therefore, a 12-month study was conducted to (i) investigate the occurrence of enteric bacteria (najs) contamination in water and fish and the variation of quality and quantity of enteric bacteria in water and fish in the Kuantan River, (ii) elucidate the relationship between water quality, and the quality and quantity of enteric bacteria in water and (iii) make recommendation based quality and quantity of enteric bacteria in water and fish. For the qualitative and quantitative estimation of bacterial load including enteric bacteria, 3 fish species namely chemparus (*Cyclocheilichtys apogon*), kawan (*Labiobarbus festivus*) and kerisi (*Pristipomoides filamentosus*) were collected by gill net from Kuantan River. Water and sediment samples were taken at 4 (four) zones: 5 km from the Kuantan River estuary insight sea, Kuantan River estuary, 5 km upstream from Kuantan River estuary and 10 km upstream of the Kuantan River estuary. Bacteria were cultured in both nutrient and marine agar plates. A molecular method (16S rRNA gene sequencing) was applied to identify all bacteria up to their genus/species level. Overall, chemparus ($6.68\times10^3 \pm 0.74\times10^3$/g) had significantly higher ($P<0.01$) bacterial load compare to kawan ($5.12\times10^3 \pm 0.60\times10^3$/g) and kerisi ($5.20\times10^3 \pm 1.49\times10^3$/g). Bacterial load was significant higher ($P<0.01$) in gut than in gill and followed by skin of fishes. No serious pathogenic enteric bacteria were observed in fishes and therefore, fish are still safe for human consumption. Enteric bacteria were commonly observed in sediments and various layers of water column in all sampling zones. This gives evidence that Kuantan river water was contaminated with najs (filth). Mean bacterial load in water in Zone D was 5.8, 4 and 4.3 times higher than in Zone A, Zone B and Zone C, respectively. A significant negative

relationship ($r = -0.742$, $P<0.01$) was observed between salinity and bacterial load in the river water. Bacterial abundance in sediment in Zone D ($8.38\times10^6 \pm 0.31\times10^6$/5g) was significant higher ($P<0.01$) than bacterial abundance in sediment in all other Zones (Zone A: $1.27\times10^6 \pm 0.31\times10^6$/5g, Zone B: $2.38\times10^6 \pm 0.61\times10^6$/5g and Zone C: $0.64\times10^6 \pm 0.35\times10^6$/5g). The mean dissolved inorganic nitrogen (DIN) concentration was significantly ($P<0.05$) lower in the monsoon months compared to other months. The mean DIN concentration in water in the sampling Zone near the Kuantan city (2.24±1.48 mg/l) was significantly higher than the mean DIN concentration (0.20±0.14 mg/l) in the upstream zone. Waste from Kuantan city is significantly influencing the growth of enteric bacterial (in water and sediment) and the water quality of the Kuantan River. The result of the study can be used for the proper management of the Kuantan River. Careful waste management by the Kuantan city authority may reduce enteric bacterial load in water and sediment and concentration of inorganic nitrogen in the Kuantan river water. These will help in increasing fish production and save the biodiversity of the river.

Key words: Fish, Water, Kuantan River, Enteric Bacteria, Water Quality

Introduction

Fish is an important for human diet in Malaysia. Presently, fish supply about 60–70 percent of total animal protein for Malaysian peoples. It is preferred as common human food because of high nutritional value. Fish fats are rich in long chain fatty acids including omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Fish protein has a high proportion of essential amino acids in a highly digestible form. Besides nutritional value, fish and fish products play a very important role against many diseases. For example, fish fats has beneficial effects on coronary heart disease (Harris and Shacky, 2004), cancer (Gerber et al., 2005) and inflammatory disease (Belluzi, 2001). Some fish proteins protect against the development of diet-induced insulin resistance (Pilon et al., 2011). However, fish could also become a source of enteric bacteria. Besides, during handling and processing, fish can be contaminated by enteric bacteria through contaminated water. Presence of enteric bacteria in the fish indicates the contamination of najs mutawassitah in fish. In addition to this, contaminated fish can become a serious threat to the public health. Likewise, contaminated water can indirectly become a threat for halal fish and public health.

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The Kuantan River is an important river in Kuantan in term of recreation, ecology and fish supply. This river has a wide variety of fishes, which is regularly marketed for human consumption. However, this River receives a lot of untreated sewage waste, which may carry enormous level of enteric bacteria. However, the presence of enteric bacteria in water and fish of the Kuantan River have not been studied yet. Therefore, the present research highlights the occurrence of enteric bacteria in water and fish of the Kuantan River. So, in order to get fish free from contamination of najs, proper management guidelines can be established after conducting the proposed research.

Background

According to Shari'ah law, Halal food should be free from najs (Bakar, 2012). Najs is an Arabic word, which refers to anything that is filthy or unclean and generally classified into three categories: najs mughallazah (sever filth), najs mutawassitah (medium filth) and najs mukhaffafah (light filth). Since najs mutawassitah originally comes from unclean or filthy matters (e.g., animal faces), it contains considerable amounts of enteric bacteria (Bakar, 2012). However, presence of enteric bacteria (bacteria which generally occur in the animal intestine) in the food gives evidence of contamination of najs mutawassitah in food. Many enteric bacteria are pathogenic and encounter a variety of health risk. For example, *Salmonella, Shigella, Campylobacter, Aeromonas, Escherichia coli,* and *Yersinia* spp. are the potential agents of diarrhea (Sudhanandh et. al., 2012).

Objectives

1) To investigate the occurrence of najs (enteric bacteria) contamination in water and fish and the variation of quality and quantity of enteric bacteria in water and fish of the Kuantan River.
2) To elucidate the relationship between water quality, and the quality and quantity of enteric bacteria in water.
3) To make recommendation based on monthly variation of quality and quantity of enteric bacteria in water and fish in order get fish free from contamination of najs.

Methodology

For the qualitative and quantitative estimation of bacterial load including enteric bacteria, fish, water and sediment samples were taken at 4 zones: Zone A (5 km from the Kuantan River estuary insight sea, Zone B (Kuantan River estuary), Zone C (5 km upstream from

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Kuantan River estuary) and Zone D (10 km upstream of the Kuantan River estuary). Fishes were collected using gill net and water samples was collected using water sampler. Fish, water and sediment samples were transferred immediately to sterilized container. Triplicate sampling and analysis was performed at each zone. Three different species of fish namely kerisi (*Pristipomoides filamentosus*), chemparus (*Cyclocheilichtys apogon*) and kawan (*Labiobarbus festivus*) collected from Kuantan River to estimate their bacterial load. The fish samples were swabbed by using sterile cotton tips at two different parts: skin and gill. Inoculating loop was used for swabbing water samples. Bacteria were cultured in both nutrient and marine agar. To avoid bacterial colonies overcrowding on the agar plates, several trials were made to find the best technique of swabbing. A molecular method (16S rRNA gene sequencing) was applied to identify all bacteria up to their genus/species level.

A series of water quality parameters (temperature, dissolved oxygen, pH, nitrate (NO_3), nitrite (NO_2) and total ammonia nitrogen (TAN) were determined every month at each sampling station. Temperature, dissolved oxygen, pH and salinity were recorded directly in the field using a portable Hydrolab equiptment (Hydrolab Minisonde® water quality multiprobes Water samples were taken from three layers (surface, middle and bottom) in each sampling station for determining, nitrate, ammonia, phosphate and silicate. They were determined according to Persons et al. (1984). All data were analyzed applying appropriate statistics using the statistical package SPSS.

Findings

All cultivable identified bacteria in three different fish species are presented in Table 1. A total 12 different cultivable bacteria species were identified from three different species of fish. Overall, chemparus ($6.68\times10^3 \pm 0.74\times10^3$/g) had significantly higher ($P<0.01$) bacterial load compare to kawan ($5.12\times10^3 \pm 0.60\times10^3$/g) and kerisi ($5.20\times10^3 \pm 1.49\times10^3$/g) (Figure 1). Bacterial load in kawan and kerisi were statistically same ($P>0.05$). Bacterial load was significant higher ($P<0.01$) in gut ($6.62\times10^3 \pm 0.78\times10^3$/g) than in gill ($5.78\times10^3 \pm 1.01\times10^3$/g) and followed by skin ($4.60\times10^3 \pm 0.96\times10^3$/g) of fishes (Figure 2).

All identified bacteria in different levels of water column (surface, middle and bottom) in four zones in the Kuantan River are presented in Table 2. Enteric bacteria were observed in water from various layers of water column in all sampling zones. This gives evidence that Kuantan river water is contaminated with najs (filth). This might be due to discharge of Kuantan city sewage into the Kuantan River. Kuantan city sewage regularly

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Table 1. Culturable bacteria identified from skin, gill and gut of kerisi, chemparus and kawan collected from Kuantan river.

Parameters	Chemparus	Kawan	Kerisi
Skin	*Enterobacter ludwigii* *Kocuria rhizophila* *Leucobacter chromiireducens* *Staphylococcus saprophyticus*	*Enterobacter ludwigii* *Kocuria rhizophila* *Leucobacter chromiireducens* *Staphylococcus saprophyticus*	*Escherichia sp.* *Enterobacter ludwigii* *Macrococcus caseolyticus* *Kocuria rhizophila* *Leucobacter chromiireducens*
Gill	*Escherichia sp.* *Enterobacter ludwigii* *Psychrobacter faecalis* *Macrococcus caseolyticus*	*Leucobacter chromiireducens* *Staphylococcus saprophyticus*	*Escherichia sp.* *Staphylococcus saprophyticus* *Enterobacter ludwigii* *Macrococcus caseolyticus* *Psychrobacter faecalis*
Gut	*Escherichia sp.* *Psychrobacter faecalis* *Enterobacter asburiae* *Macrococcus caseolyticus*	*Psychrobacter faecalis* *Macrococcus caseolyticus* *Bacillus tequilensis* *Bacillus licheniformis* *Bacillus aerophilus*	*Psychrobacter faecalis* *Staphylococcus saprophyticus* *Enterobacter cloacae*

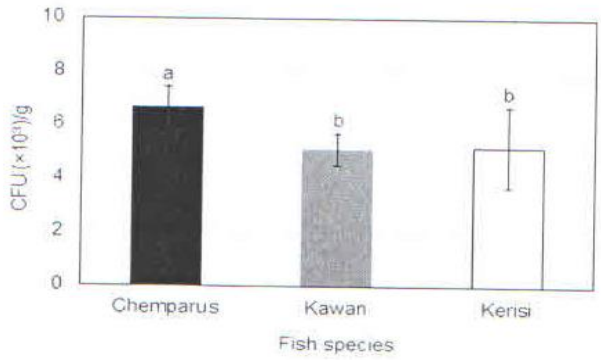

Figure 1. Mean (bar: ±SD) number of cultivable bacteria (CFU/g) in Chemparus, Kawan and Kerisi. Mean with no letters in common are significantly different ($P<0.05$).

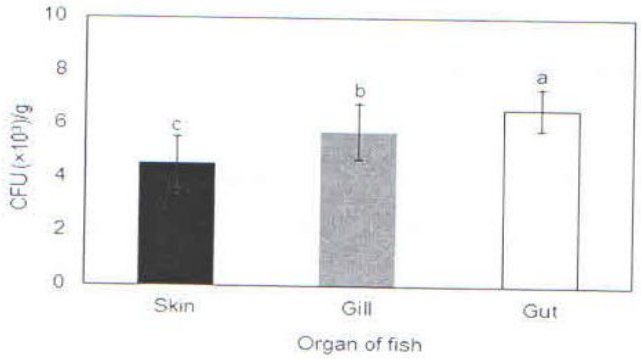

Figure 2. Mean (bar: ±SD) number of cultivable bacteria (CFU/g) in skin, gill and gut of fishes. Mean with no letters in common are significantly different ($P<0.05$).

discharges waste water containing organic matter including animal excreta. Mean bacterial load in water in Zone D ($11.83\times10^6 \pm 1.15\times10^6$/ml) was higher ($P<0.01$) than in water in other zones (Zone A: $2.04\times10^6 \pm 0.49\times10^6$/ml, Zone B: $2.99\times10^6 \pm 0.47\times10^6$/ml and

Table 2. Bacteria species identified from surface, middle and bottom layers of water column in various location in the Kuantan River water.

Zone	Surface	Middle	Bottom
A	*Algoriphagus zhangzhouensis* *Citromicrobium bathyomarinum* *Cronobacter sakazakii* *Enterobacter sp.* *Escherichia coli* *Methylobacterium hispanicum* *Pseudoalteromonas espejiana* *Pseudomonas sp.* *Psychrobacillus psychrodurans* *Staphylococcus arlettae* *Vibrio fluvialis*	*Citromicrobium bathyomarinum* *Cronobacter sakazakii* *Enterobacter sp.* *Escherichia coli* *Pseudoalteromonas espejiana* *Pseudomonas sp.* *Staphylococcus arlettae* *Vibrio fluvialis* *Vibrio neptunius*	*Cronobacter sakazakii* *Enterobacter sp.* *Escherichia coli* *Methylobacterium hispanicum* *Pseudoalteromonas espejiana* *Shigella boydii* *Vibrio neptunius*
B	*Alcaligenes faecalis* *Bacillus cereus* *Enterobacter sp.* *Escherichia coli* *Pseudoalteromonas espejiana* *Staphylococcus arlettae* *Staphylococcus haemolyticus* *Vibrio fluvialis*	*Bacillus cereus* *Cronobacter sakazakii* *Enterobacter sp.* *Enterobacter sp.* *Escherichia coli* *Idiomarina baltica* *Pseudoalteromonas espejiana* *Pseudomonas sp.* *Serratia marcescens* *Staphylococcus arlettae* *Vibrio fluvialis*	*Bacillus cereus* *Enterobacter sp.* *Enterobacter sp.* *Escherichia coli* *Pseudoalteromonas espejiana* *Vibrio fluvialis* *Vibrio neptunius* *Virgibacillus sp.*
C	*Enterobacter cloacae* *Escherichia fergusonii* *Idiomarina baltica* *Lysinibacillus xylanilyticus* *Proteobacterium* *Providencia vermicola* *Pseudoalteromonas espejiana* *Pseudomonas sp.* *Psychrobacillus psychrodurans* *Thioclava pacifica* *Uncultured bacterium* *Vibrio alginolyticus* *Vibrio fluvialis*	*Alcaligenes faecalis* *Cronobacter sakazakii* *Enterobacter cloacae* *Escherichia coli* *Escherichia fergusonii* *Idiomarina baltica* *Lysinibacillus xylanilyticus* *Providencia vermicola* *Salmonella sp.* *Thioclava pacifica* *Uncultured Clostridiales* *Vibrio brasiliensis* *Vibrio fluvialis* *Virgibacillus sp.*	*Cronobacter sakazakii* *Enterobacter cloacae* *Escherichia coli* *Escherichia fergusonii* *Idiomarina baltica* *Lysinibacillus xylanilyticus* *Proteobacterium* *Providencia vermicola* *Psychrobacillus psychrodurans*
D	*Burkholderia cenocepacia* *Erythrobacter sp.* *Escherichia coli* *Escherichia sp.* *Pantoea agglomerans* *Photobacterium ganghwense* *Pseudomonas monteilii* *Pseudomonas oleovorans* *Pseudomonas plecoglossicida* *Thalassospira sp.*	*Bacillus cereus* *Escherichia coli* *Escherichia fergusonii* *Lysinibacillus fusiformis* *Microbacterium paraoxydans* *Oceanisphaera psychrotolerans* *Photobacterium ganghwense* *Pseudomonas monteilii* *Thalassospira sp.* *Vibrio fluvialis*	*Azospirillum sp.* *Bacillus cereus* *Erythrobacter sp.* *Escherichia fergusonii* *Loktanella sp.* *Lysinibacillus fusiformis* *Microbacterium paraoxydans* *Oceanisphaera psychrotolerans* *Pantoea agglomerans* *Photobacterium ganghwense* *Pseudomonas monteilii* *Pseudomonas plecoglossicida* *Thalassospira sp.* *Vibrio fluvialis*

Zone C: $2.43\times10^6 \pm 0.45\times10^6$/ml) (Figure 3). These results concur with Hoch and Kirchman (1993) and Painchaud et al. (1996), who reported almost similar bacterial load in river estuaries. Bacterial load in water in Zone B and Zone C are statistically same ($P>0.05$).

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Bacterial load in water in Zone D was significantly lower ($P<0.05$) compared to bacterial load in water in all other Zones. Mean bacterial load in water of Zone D was 5.8, 4 and 4.3 times higher than Zone A, Zone B and Zone C, respectively. This indicates that bacterial increased with decreased salinity. A significant negative relationship ($r = -0.742$, $P<0.01$) was observed between salinity and bacterial load in the water. This finding concurs with Painchaud et al. (1996), who also observed increasing trend of bacterial load with decreasing salinity.

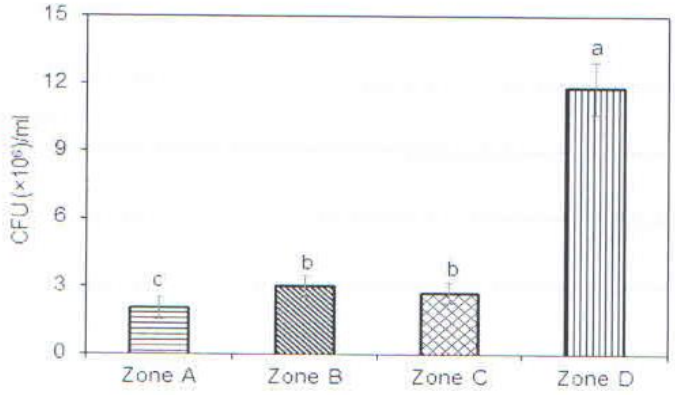

Figure 3. Mean (bar: ±SE) number bacteria (CFU/ml) observed in water in various zones in the Kuantan river. Mean with no letters in common are significantly different ($P<0.05$).

The bacterial load in various layers of water column are presented in Figure 4. The bacterial load in water near bottom ($6.10\times10^6 \pm 1.76\times10^6$/ml) was significantly ($P<0.05$) higher than the bacterial load in the middle layer of water column ($3.83\times10^6 \pm 0.98\times10^6$/ml). Bacterial load in water near bottom and bacterial load in surface layer of water ($4.86\times10^6 \pm 1.10\times10^6$/ml) were statistically same ($P>0.05$). Similarly, the bacterial load in surface layer of water and the bacterial load in the middle layer of water were similar ($P>0.05$).

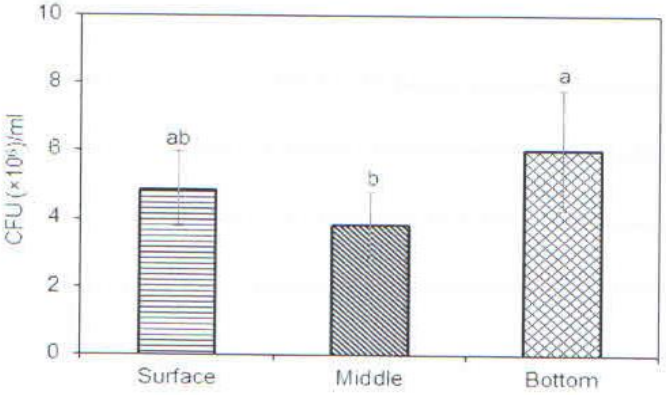

Figure 4. Mean (bar: ±SE) number bacteria (CFU/ml) observed in various layer of water column in the Kuantan river. Mean with no letters in common are significantly different ($P<0.05$).

All identified bacteria in the sediment in different sampling Zones in the Kuantan River are presented in Table 3. Sediments in Zone A, Zone B, Zone C and Zone D had 11, 7, 10 and 10 species of bacteria, respectively. Enteric bacteria species were observed in sediment in all Zones. Bacterial abundance in sediment in Zone D ($8.38\times10^6 \pm 0.31\times10^6$/5g) was significant higher ($P<0.01$) than bacterial abundance in sediment in all other Zones (Zone A: $1.27\times10^6 \pm 0.31\times10^6$/5g, Zone B: $2.38\times10^6 \pm 0.61\times10^6$/5g and Zone C: $0.64\times10^6 \pm 0.35\times10^6$/5g) (Figure 5). Bacterial abundance in sediment in Zone A, Zone B and Zone C are statistically ($P>0.05$) same.

Table 3. .Bacteria identified in sediment samples collected from different Zones in the Kuantan river.

Zone A	**Zone B**
Serratia marcescens	*Bacillus infantis*
Bacillus infantis	*Brevibacillus reuszeri*
Enterobacter sp.	*Enterobacter sp.*
Escherichia coli	*Escherichia coli*
Microbacterium paraoxydans	*Pseudoalteromonas espejiana*
Pseudoalteromonas espejiana	*Vibrio neptunius*
Psychrobacillus psychrodurans	*Virgibacillus sp.*
Serratia marcescens	
Thioclava pacifica	
Vibrio neptunius	
Virgibacillus sp.	
Zone C	**Zone D**
Bacillus cereus	*Bacillus cereus*
Bacillus infantis	*Bacillus drentensis*
Enterobacter sp.	*Bacillus pumilus*
Escherichia coli	*Escherichia coli*
Escherichia fergusonii	*Escherichia fergusonii*
Idiomarina baltica	*Oceanisphaera psychrotolerans*
Microbacterium paraoxydans	*Photobacterium ganghwense*
Pseudomonas pachastrellae	*Pseudomonas oleovorans*
Psychrobacillus psychrodurans	*Thalassospira sp.*
Thioclava pacifica	*Vibrio fluvialis*

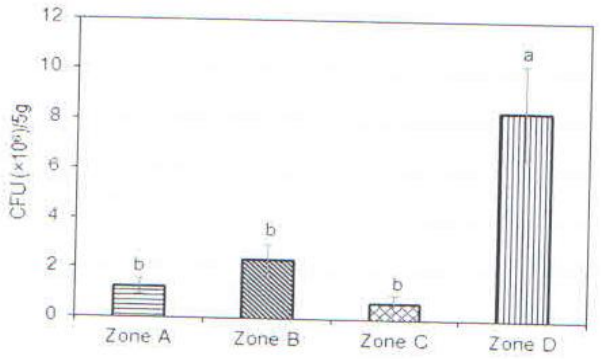

Figure 5. Mean (bar: ±SE) number bacteria (CFU/5g) observed in sediment at various sampling Zones in the Kuantan river. Mean with no letters in common are significantly different ($P<0.05$).

The overall effects of sampling month, sampling zone, water depth and their interactions on water quality parameters are presented in Table 4. Temporal effect was significant ($p<0.01$) on all water quality parameters. Similarly, sampling zone had significant effects on all water quality parameters except nitrite concentration in water. Except pH, nitrite and nitrate, all water quality parameters were significantly different ($p<0.05$) in different layers of the Kuantan river water column (Table 4). Overall mean DIN concentration was significantly higher ($P<0.05$) in downstream sampling zones compared to upstream sampling zones. However, DIN concentration in downstream water might be influenced by the waste from the Kuantan city waste. Higher DIN concentration in downstream water might also be influenced by higher salinity as there was significant positive relationship ($r= 0.66$, $p=0.01$) between ammonia concentrationand salinity in the Kuantan river water (Figure 6). In the present study, the mean total DIN concentration was highest in water near the river bottom (2.06±0.15 mg/l), followed by middle (1.81±0.13 mg/l) and surface layer (1.70±0.11 mg/l) of water column.

Table 4 Effects of month, sampling zone, level (surface, middle and bottom) and their interaction on physico-chemical parameters in Kuantan River based on two-way repeated measure ANOVA.

Variable	Mnoth	Zone	Level	Month×Zone	Month×Level	Zone×Level
Temperature (°C)	**	**	**	**	**	**
Salinity (ppt)	**	**	**	**	**	**
Conductivity	**	**	**	**	**	**
p^H	**	**	NS	**	NS	**
DO (mg/l)	**	**	**	**	**	NS
Nitrite (mg/l)	**	NS	NS	**	NS	NS
Nitrate (mg/l)	**	**	NS	**	NS	NS
Ammonia (mg/l)	*	**	**	**	NS	NS
DIN (mg/l)	**	**	*	**	NS	NS

*P< 0.05, **P< 0.01; NS: not significant.

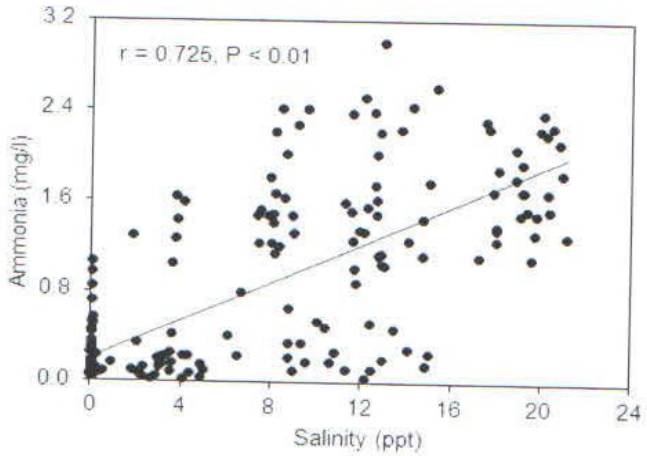

Figure 6. Relationship between ammonia concentration and salinity in the Kuantan river water.

Conclusion

This is the first study that evaluates the occurrence of najs (enteric bacteria) contamination in water, sediment and fish in the Kuantan River and elucidate the relationship between water quality, and the quantity of enteric bacteria in water. This study shows that bacterial load in fish, sediment and water, and the dissolved inorganic nitrogen concentration in the river water varies on location and depth of water column. No serious pathogenic enteric bacteria were observed in fishes and therefore, fish are still safe for human consumption. However, several serious pathogenic enteric bacteria in high quantity were observed in water and sediment in the Kuantan River. Waste from Kuantan city and salinity are two important factors that are influencing bacterial load in water, fish and river sediment, and ammonia concentration in water of the Kuantan River. The result of the study can be used for the proper management of the Kuantan River. Careful waste management by the Kuantan city authority may reduce enteric bacterial load in water and sediment and help in maintaining water quality in a suitable range. These will help to increase the safety level to consume Kuantan river fishes and save the biodiversity of the Kuantan River.

Output

1. 4 articles have been submitted to publish in citation indexed journals.
2. 1 Book Chapter (will be published in book by springer) entitled "DNA Barcoding of Indigenous Bacteria in Selected Fishes from a Tropical Tidal River" will be submitted by 15 January 2017 (the title already accepted by editor).
3. 4 Final Year Project theses have already been produced.
4. 3 conference presentations: (abstracts are available in abstract books).
5. Besides these, the project has produced a lot of data which are going to be published soon as indexed journals articles.

Future Plan of the research

Besides fish and bacteria, plankton and benthic macroinvertebrates are also important for the growth of fish and water quality. Future research should focus on how phytoplankton, zooplankton and benthic macroinvertebrates are related with bacteria load (including enteric bacteria) and fish growth in the Kuantan River ecosystem.

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