Characterization of culturable bacterial communities associated with cage cultured fish under different stocking densities in Setiu Wetlands, Terengganu, Malaysia.

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Research note

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Abstract

The study objective was to evaluate the effects of fish stocking density on the prevalence of pathogens isolated from sixty clinically healthy fishes reared under different densities in floating net-cages in Setiu lagoon. The water temperature, pH, DO, TDS, salinity, water clarity, depth, and coliform concentration, were all determined within the fish cages. The healthy fish samples were randomly collected from 3 sites in cages with low and high stocking densities at each site. The bacteria were isolated from the skin, gills, kidneys, and liver of each fish sample, followed by identification to species level using the VITEK-2 system.

The pathogens with beta-hemolysis characteristics were selected for antibiotic susceptibility against the following drugs: AM-10 µg, P-10 U, CL-30 µg, TC-30 µg, CP-5 µg, GM-10 µg, KM-30µg, and SM-10 µg. Water quality parameters showed no differences between the cages of low and high fish densities except for site C.

The total number of isolates, microbial species, and the number of pathogens isolated from fish revealed no significant difference between the fish stocked in low and high densities. A total of 25 bacterial species were isolated, which included 14 gram-positive and 11 gram-negative. The (SM) drug application is suspected on this farm.

1. Introduction

Nowadays, as the world population is expanding, the demand for food is increasing especially for fish consumption. Therefore, solutions have been introduced mainly by intensive aquaculture systems that have become the point of economic interest of many farmers. In the case of Setiu Wetlands, Malaysia, this area has a significant potential for both aquaculture activities and fisheries such as floating net cages that have become one of the fastest flourishing economic activities. Stocking large quantities of biomass/fish numbers with larger feed inputs in the fish ponds allows more financial income, but it can eventually lead to disease outbreaks (Duarte et al., 2019; Das et al., 2019). Wang et al., (2018) have indicated that intensive stocking fish densities in cage cultures increase the competition for food, living space and social interactions between the fish individuals, which is a food ration hierarchy (e.g., fish at the cage-bed get less food), and aggressive behaviors between fish individuals.

Moreover, it causes lowering of individual fish growth and increasing cortisol (stress hormone) levels (Wang et al., 2019; Wang et al., 2019). The latter is the physiological factor responsible for immunosuppression, i.e., it reduces the immune competence to respond to harmful agents such as pathogens. In addition, a common problem is fish compression at the bed of the cage culture due to gravitational fish weight, which can cause abrasions and also injuries by spines of the adjacent fish (Conte, 2004).

The intensive production of fish in cages might induce deleterious effects on the ambient aquatic environment such as water pollution resulting from nutrient leaching of uneaten fish feed and fish excreta inputs from cages to the surrounding environment makes the cage farms as an extra source of nutrients that encourage microbial growth. thus, poor water quality is considered a source of stress that reduces the health of cultured fish and makes them more susceptible to bacterial infections (Zamri-Saad et al., 2014).

As an approach for disease management, fish producers tackle such problems by removing the infected and dead fish from the cages and by the frequent use of antibiotics to treat diseases (Chitmanat et al., 2016), as well as for prophylactic purposes for the healthy fish (Miller & Harbottle, 2018). Unfortunately, the frequent use of the antibiotics contributes to lower the immune defense, through disturbances of the host-microbiota, increases the risk emergence of antibiotic-resistant bacteria in the environment and the host animal microbiome (López et al*.,* 2018), and the presence of antibiotic residues in fish tissues (Zamri-Saad et al., 2014; Chitmanat et al., 2016).

There are viable alternative procedures that have been developed as primary preventive measures to control disease problems in fish farms, e.g., the use of immunostimulants comprises of) vitamins A, C, and E (Wang et al., 2016); herbal extracts, prebiotics, and probiotic bacteria (Assefa & Abunna, 2018). The latter is known to reinforce nonspecific fish immunity. While fish vaccination is a prophylactic method used to improve the acquired immunity of the fish and protect them against specific disease outbreaks, also its economically-important in aquaculture that helps in decreasing the use of antibiotics.

Lastly, the application biosecurity strategies on fish farms are mainly represented in the proper quantities of food rations, clean feed source, cleaning or changing the net-cages, disposal of dead fish away from the riverbanks (Chitmanat et al., 2016), avoidance of high stocking densities (Assefa & Abunna, 2018), use of clean equipment, limited fish transfer between cages, monitoring of vector prevalence (e.g., birds and rodents), and also when handling the fish (e.g., clean human hands or wearing protective gloves).

This study was undertaken to evaluate the effects of fish stocking density on the prevalence of pathogens isolated from fish reared in commercial floating net-cages in Setiu Wetland and assessing their antibiotic susceptibility profiles. We assumed that fish under high stocking density requires high feed inputs that in turn will generate high levels of excreta and food leaking, which affect the water quality parameters and promote bacterial growth; and need high amounts of antibiotic use (e.g., prophylactic purpose) which in turn result in more resistant bacterial strains.

The results of the present study can provide us insight into information about the sustainability of the farm during a critical period of environmental stress, especially when the temperature is 30 °C and above in dry season.

2. Materials And Methods

2.1 Study area

The study was conducted at a private commercial fish culture farm located in Setiu Wetland, along the north coast of the Terengganu state, Malaysia facing the South China Sea (Figure 1).

2.2 Culture cages

In this study, the fish farm has a total of 50 floating net-cages filled two fish species separately; the tiger grouper (*Epinephelus fuscoguttatus*) and the Asian seabass (*Lates calcarifer*). Both varied in age group and numbers per cage per sampling site, the latter species was the most abundant in this farm due to its low economical price. These cages came in two different depths (1.7 m in sites A and C, while 1.9 m in site B). The fish stocking density was determined by the number of fish per cage of the three-dimensional medium (Conte, 2004). The fish density was determined, by pulling submerged nets into separate quadrants and visually determining their density to confirm the stocked fish numbers that were indicated by the farm owners. The latter continuously replenish the cages with new fish after they harvest the fish to sell it to the local markets (personal communication).

The small cages contained a low fish density (Table 1), except for the cages at site A2. Their dimensions were 4.9 m (length) x 3 m (width) x 1.7 m (depth), with a total volume of 24.99 m^3, for the cages at the sites A1, A2, and C1, and 27.93 m^3 at the site B1. The high fish density was contained in small cages at the site A2, and in big cages: 6 m (length) x 3 m (width) x 1.7 m (depth) elsewhere. Their volume was 30.6 m^3 at the site C2 and 34.2 m^3 at the site B2.

The fish were fed two times a day with a commercially manufactured feeds pellet (Poly fish feed, Star feed, Malaysia). The total number of stocked fish per sampled cages was between 300 – 1000 fish; and the selected fish samples for this study were within the marketable weight (700 gm – 1000 gm).

2.3 Fish Sampling

The fish samplings were conducted in July 2018 during the dry season from three sites A, B, and C (see Figure 1). The fish were randomly collected with a total of sixty (60), consisted of 20 tiger grouper and 40 seabass from cages of 2 different fish densities at each site. Gross examination indicates that all fishes were healthy. According to the farm history, there were no disease outbreaks on this farm.

Table 1. Study sampling design, fish species, and their densities among the sampling sites.

Cages	Total fish no. stocked per net-cage	Stocking density (fish/m^3)	Sampled Fish	Fish species	Sampling sites	GPS coordinates
	300	12	10	Grouper	*A1	N 05°40'59.6" E 102°42'40.7"
Low fish density	400	14.32	10	Seabass	[a] B1	N 05°40'54.5" E 102°42'42.2"
	600	24	10	Seabass	*C1	N 05°40'45.1" E 102°42'50.3"
	600	24	10	Grouper	*A2	N 05°40'59.5" E 102°42'40.7"
High fish density	1000	29.23	10	Seabass	[w] B2	N 5°40'54.5" E 102°42'41.4"
	1000	32.68	10	Seabass	[b] C2	N 05°40'45.6" E 102°42'50.0"
Total	3600	136.23	60			

The volume of submerged net-cages in the water: * = 24.99 m^3, [a] = 27.93, [b] = 30.6 m^3, [w] = 34.2 m^3.

2.3.2 Measurements of water quality parameters and GPS

Temperature, pH, dissolved oxygen (DO), total dissolved solids (TDS) and salinity, were all determined using a portable device YSI multiprobe sensor (YSI, 556 MPS, USA). Water transparency was determined using a Secchi disk (Wildlife Supply Company, USA). All parameters were measured within the cages before, during and after the fish sampling; from three different depths (below the water surface, 1 m and 2 m) Besides, the water flow rate was assessed using a current water meter (Model: Valeport, UK), and the depth of the water column was measured by an ultrasonic depth sensor (Speed Tech SM-5, USA) at the three sampling sites.

The instrument sensors were calibrated according to the manufacturer's recommendations before field sampling. GPS coordinates of the sampling sites were taken using a Garmin GPS device (SNAP 276C, US). The map of the study area was created by using the following programs: Google Earth V. 7.3 and ArcMap V. 10.3.

2.3.3 Water sampling and analysis of microbiological quality

Water samples of yellowish-brown color were collected in sterile plastic containers of 50 ml within the fish sampling cages, at a depth of 15-20 cm below the water surface due to the shallowness of water in the study area (less than 2 m max deep) (Table 2). The fecal contamination in the water samples was analysed using the most probable number (MPN) method for coliform bacteria according to the WHO guidelines (Bartram & Rees, 2000; Bumadian et al., 2013, Mishra et al., 2018).

2.4 Bacterial isolation and identification methods

The fish was selected based on its healthy appearance and further observed for any signs of common diseases such as abdominal swelling, external ulcers, hemorrhages along the skin, gills, eyes, and fins. Also during the dissecting procedure, the examination was extended to the kidneys and liver, which was done according to the published guidelines by Nagasawa & Cruz-Lacierda, (2004).

The bacteria were isolated in three replicates of the outer parts (skin and gills) and internal parts (liver and kidney) of each fish sample, plated on three media; Zobell Marine Agar (ZMA) (HIMEDIA, India), Thiosulphate-Citrate Bile Salts Sucrose (TCBS) agar (Difco™, USA), ready to use Blood Agar plates (Thermo Scientific Microbiology Sdn Bhd, Malaysia). The instructions of the inoculation method were followed from

The instructions of inoculation method were followed the Public Health England, standards for microbiology investigations (UK, SMI, 2017).

All the inoculated media were incubated at 37˚C for 24 hours. The bacterial colonies that grew were sorted into different types according to the colony characteristics such as shape, size, color, structure, edge, elevation, and the opacity. After successive sub-culturing onto additional media of (ZMA) and (TCBS)-agar, pure colonies were obtained.

The purified isolates were subjected to conventional simple tests such as oxidase and catalase tests. Further, the isolates were stained by Gram staining procedures then were divided into two groups of gram positive and negative bacteria to specify the appropriate cards type used in the microbial identification VITEK®2 System (BioMérieux, USA) and identification process was carried out to species level using an automated bacterial identification instrument (VITEK-2 Compact System, BioMérieux, USA).

2.5 Hemolytic activity

The pure isolates were tested for hemolytic activity according to the guidelines from Chow et al., 1983. was done by transferring a loopful of freshly cultured bacteria on (ZMA) and streaked onto Blood agar plates (made of 5% of sterile fresh sheep blood) for 24 hours at 37°C. The observed clear zones of hemolysis around the colonies indicate the ability of tested bacteria to hemolyze erythrocytes.

2.6 Antibiotic susceptibility

To avoid inappropriate antimicrobial agents for this experiment and to isolate resistant strains, the fish farmers were asked (personal communication) whether they use any antibiotic supplement: they claimed not using anything else than feed pellets.

A range of antibiotics was selected based on three criteria namely the most commonly used in aquaculture and human therapy (Scarano et al., 2014); the annual reports of the World Organisation for Animal Health regarding general antimicrobial agents used in aquatic food-producing animals (OIE, 2018); and their inhibition mechanisms (modes of action) against bacterial strains.

The hemolytic bacterial isolates were tested for antibiotic susceptibility against eight (8) antibiotics (Oxoid®, UK), (see Table 3).

Table 3. The antimicrobials selected for the susceptibility test of isolated pathogenic bacteria.

No.	Gram positive	Gram negative
1	Chloramphenicol 30 µg	Gentamicin 10 µg
2	Ampicillin 10 µg	Ciprofloxacin 5 µg
3	Penicillin 10 units	Streptomycin 10 µg
4	Tetracycline 30 µg	Kanamycin 30 µg

The susceptibility test was done using the Kirby-Bauer disk diffusion method on (MHA) to determine the resulting inhibition zones of the tested antibiotic against the isolated pathogen. The bacterial inoculum preparation followed the “direct colony suspension method” according to guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, 2002).

The sensitive isolates were classified as susceptible, or intermediate according to the published standardized breakpoint values in the guides (NCCLS, 2002; CLSI, 2015; 2017) except for the streptomycin antibiotic (breakpoint values for other organisms) was determined from (https://bio.libretexts.org) see Table 4.

A quality control test for drug susceptibility testing was done for the selected antibiotics using environmental strains of *Escherichia coli* and *Bacillus cereus*; that were obtained from microbiology lab at the Institute of Marine Biotechnology, Universiti Malaysia Terengganu.

2.7 Data analysis

The data analysis was performed using the analytical software R version 3.3.2, packages used in R were: psych, and stats.

Non-parametric statistical analysis was used because the dataset did not meet the assumptions of normality distribution nor homogeneity of variance even after transformation methods. The Kruskal–Wallis test was done to compare the differences of the variables among the sampling sites; and the unpaired two-tailed Wilcoxon–Mann–Whitney test was used to make a pairwise comparison test with Bonferroni correction to identify the significant differences between each variable within and among each sampling site.

The tested variables were between cages with low and high fish densities: (i) the water physicochemical parameters; (ii) the total number of bacterial isolates; (iii) the total number of pathogen isolates; and (iv) the

total number of pathogenic species.

The Spearman's rank correlation coefficient test (rho) was used to determine the possible correlations between the water quality parameters (physicochemical and microbiological), fish density, and the total number of microbial isolates.

The antibiotic susceptibility data were not normally distributed, homogeneous nor had an equal sample size between groups of the isolated pathogens from low and high fish densities. Thus, we determined the median values of the bacterial susceptibility profiles to test for the significance and plotted the means and standard error (± SE) of the bacterial susceptibility profiles in bar charts to see the differences using Excel, Microsoft Corporation, 2016.

Lastly, the maximum: minimum ratio and the overall median ratio were determined to check for the variation in antibiotic susceptibility within the same bacterial species.

The significance of the statistical tests was assessed at an α of 5% probability level, and all significant differences were $p < 0.001$ (with Bonferroni correction) unless mentioned otherwise.

3. Results

3.1 Physicochemical parameters

Mean values of the physicochemical parameters with their standard deviations (±SD) at all sampling sites are presented in Table 2. Statistical analyses show that all water quality parameters (temperature, DO, TDS, salinity, pH, and Secchi depth) were not significantly different between the samples taken from the cages (A1 and A2) of (12 and 24 fish/m^3) nor between the samples taken from the cages (B1 and B2) of (14.32 and 29.23 fish/m^3), with the exception for the pH in both B sites and water flow rate between all sampling sites (Wilcoxon, $P < 0.05$).

The sites (C1 and C2) of (24 and 32.68 fish/m^3) showed a significant difference (Wilcoxon, $P < 0.05$) in all water parameters except for DO and Secchi depth readings.

However, there was a significant difference in the observed values of water samples between the sampling sites (A, B and C) for each parameter (Wilcoxon, $P < 0.05$), except for the water transparency.

The water sample from C2 has recorded the highest temperature and lowest TDS, salinity, pH, and moderate water flow in comparison to other sites. On contrary, the site A1 had the highest DO, TDS, salinity, Secchi depth, pH and the lowest water flow among the sampling sites, while B sites showed the lowest temperature with otherwise moderate water parameters.

3.2 Microbiological parameters

Total coliform (TCF) counts of the tested water samples for all sampling sites are shown in Table 5. Table 6 displays the fecal coliforms counts from the six sampling sites. A further test was done to confirm the presence of thermotolerant fecal coliforms (FC) at 44.5°C Table 7. The lowest concentration of (TCF) was observed at site A, whereas the highest concentration was recorded at site C1. The MPN value index of (FC) counts varied from 34/100 ml for the site A1 to 920/100 ml in sites B1 and C1.

3.3 Bacterial isolates

A total of 88 bacterial strains were isolated from 60 visually healthy fish with no clinical sign of bacterial infections, except one sample that consisted of congested and swollen kidneys upon necropsy, (Table 8 and Table 9).

The number of pathogenic isolates (α and β-hemolysins) was higher (86 strains) than the number of commensal strains (γ- hemolysin) (2 strains). Therefore, no statistical test was performed for differences between pathogenic and commensal isolates, but instead, we determined the frequency of occurrence for each pathogen per and among the sampling sites to check for disease status incidence in this farm.

The number of identified taxa (“species”) was 25; they corresponded to bacterial colonies isolated from the skin, gills and only one from kidneys. More specifically, they represented 24 species and one genus, which were 14 (56%) gram-positive bacteria and 11 (44%) gram-negative bacteria. The detailed information on the identification, physiological characteristics, numbers, and frequency of occurrence of each isolate are presented in Table 8 and Table 9.

There was no significant difference between the total number of isolates from the low and high fish densities among the three main sampling sites (Wilcoxon, $P > 0.05$), but there were significant differences between the sites A1 with B1, C1, C2, and site A2 with C2, (Figure 2). The total numbers of isolated species showed no significant difference between the cages of low versus high fish densities (Wilcoxon, $P > 0.05$), (Figure 3). However, the most common species isolated were *Staphylococcus warneri* (n = 14, 15.90%) followed by *Staphylococcus sciuri* (n = 13, 14.77%), *Proteus mirabilis* (n = 8, 9.1%), *Staph. pseudintermedius* (n = 6, 6.81%), *Micrococcus luteus* and *Bacillus cereus* group (n=5 isolates each, 5.68%). The bacterial species with 3 isolates each (3.41%) were *Rhizobium radiobacter* and *Sphingomonas paucimobilis*.

The total isolates revealed three types of bacterial hemolysins: Alpha (α), Beta (β), and Gamma (γ) hemolysin’s that recorded 52 (59.1%) strains, 34 (38.63%) strains and 2 (2.27%) strains respectively. The dominant bacteria of producing cytotoxins (α and β categories) (n=86, 97.73%) mostly were gram-positive (61 strains, 69.32%) compared to gram-negative ones (25 strains, 28.41%). Nonetheless, the statistical analysis revealed no significant difference (even after excluding the two gamma strains from site B1 and C1) between the pathogenic strains isolated from fish stocked in low density versus high density at the

three sampling sites (Wilcoxon, P > 0.05). A significant difference was however observed between the site C2 and the site A1 or A2, (Figure 4).

3.4 Antibiotic susceptibility

The susceptibilities of β-hemolytic bacteria (n=34) were assessed against eight different antibiotic agents then a summary is presented in Table 10.

In general, most tested isolates exhibited susceptibility to the selected drugs, (Figure 5). All tested strains of gram-positive bacteria (n=22) showed susceptibility to (CL), (AM), (P), and (TC), with the exception of strains of the group *Bacillus cereus*, which were resistant to most of the selected drugs except (CL). The *Bacillus cereus* group showed resistance of 22.73% to each (AM) and (P) and about 13.64% to (TC).

However, the resistance of some isolates was observed: *Pseudomonas aeruginosa* towards (KM) (this strain represented 8.33% of the 12 strains tested), *Vibrio parahaemolyticus* towards (SM) (8.33%), and *Rhizobium radiobacter* towards (GM) (8.33%) and (SM) (8.33%). Most of the gram-negative strains revealed intermediate susceptibility of 41.67% to (SM), (Figure 5).

Mean values and standard errors (±SE) of the diameter of the inhibitory zones are presented in Figure 6. The susceptibility profiles of the isolated pathogens from fish stocked in low density were significantly less susceptible to (CP), (GM), (KM), and (TC), except for (SM) which shown significantly lower susceptibility values for the pathogens isolated from high fish density. The antimicrobial agents: (AM), (CL), and (P) showed no significant difference because equal medians were obtained (24, 32, and 33 respectively) between both low and high susceptibility treatments (Figure 6).

The Maximum: Minimum Ratio (MMR) and the overall median ratio were calculated as a measure of variation in the levels of antimicrobial resistance for the same species of isolated pathogens. For this setting, we selected only the pathogenic bacteria that shown resistance for more than two strains, which were for the Bacillus cereus group (5 strains).

The (MMRs) and the median ratios for this bacterial group showed a high variation in the resistance profiles for the tested drugs, which were as follow: (20:8) - 14, (22:8) - 15, and (25:10) - 17.5 for the drugs (AM), (P), and (TC) respectively.

Parameters	Sampling sites					
	A1	A2	B1	B2	C1	C2
Temperature (°C)	30.29 ± 0.03 hh, b	30.31 ± 0.03 hh, g	30.17 ± 0.05 ff, b, g s	30.16 ± 0.07 ff, b, g, s	30.29 ± 0.03 hh, s, aa	30.40 ± 0.00 b, s, g, aa
DO (mg/L)	5.19 ± 0.33 ee, b	4.99 ± 0.31 ee, w	4.50 ± 0.19 bb, b, w, c	4.22 ± 0.37 bb, b, w, v	4.14 ± 0.35 cc, b, w, c, v	4.27 ± 0.23 cc, b, bb, w
TDS (g/L)	27.29 ± 0.35 cc, b	27.23 ± 0.34 cc, v, g	25.89 ± 0.39 nn, b, g, s	26.30 ± 0.50 nn, b, v, s	24.70 ± 0.08 aa, b, g, s	23.39 ± 0.08 aa, b, g, s
Salinity ppt	26.80 ± 0.39 zz, v, s	26.73 ± 0.37 zz, c, e	25.36 ± 0.46 uu, s, c, a	25.78 ± 0.62 uu, v, e, g	24.06 ± 0.28 aa, s, c, g, a	22.59 ± 0.10 aa, s, c, g, a
pH	7.95 ± 0.02 ii, b	7.95 ± 0.01 ii, s	7.82 ± 0.03 tt, b, s g,	7.86 ± 0.03 tt, b, s, p	7.65 ± 0.01 aa, b, s, g, p	7.34 ± 0.18 aa, b, s, g, p
Transparency / Secchi depth (cm)	141.33 ± 1.53 dd, k	125.00 ± 13.23 dd	118.33 ± 2.89 bb, k, m	117.33 ± 2.52 bb	96.00 ± 1.00 cc, m	100.33 ± 5.51 cc, m
Water flow rate (meter/second)	0.03 ± 0.04 ab, ac		0.19 ± 0.08 ab, bc		0.06 ± 0.05 ac, bc	
Water depth (m) in the cages	1.7		1.9		1.7	

Table 2. The mean values ± SD for the selected physicochemical water parameters among the sampling sites.

Legend: P-values interpretation: ac, bc, w, v = (0.001), e = (0.004), ab = (0.028), k, m = (0.07); the double letter superscript in the same row are pairwise comparisons between the groups (*i.e.,* A1 & A2, B1 & B2, and C1 & C2) are NOT significantly different at 5% level with exception of the following letters tt = (P=0.016), aa and any other letters per parameter (row) are pairwise comparisons between groups with a significant difference (P=0.0001).

Water samples	No. of tubes with positive reactions for each volume of raw water sample			MPN Index per 100 ml	95% Confidence limits	
	10 ml	1 ml	0.1 ml		Low	High
A1	5	5	2	540	180	1400
A2	5	5	1	350	120	1000
B1	5	5	3	920	300	3200
B2	5	5	3	920	300	3200
C1	5	5	5	>1800	□□□	□□□
C2	5	5	3	920	300	3200

Table 5. Results of the presumptive tests of total coliform bacteria detection in the water samples.

Table 6. Confirmatory test results for the presence of fecal coliforms on EMB agar medium.

Water samples	Number of positive reactions on EMB agar plates for each sample volume		
	10 ml	1 ml	0.1
A1	++++	++++	–
A2	+++++	+++	–
B1	+++++	+++++	+++
B2	+++++	++++	+++
C1	+++++	+++++	+++
C2	+++++	+++++	++

Legend: " + " represent the positive agar plate counts of fecal coliform growth isolated from the positive tubes of the presumptive test.

Table 7. Completed test results of fecal thermotolerant bacteria in lactose broth tubes.

Water samples	No. of tubes with positive reactions for each volume of raw water sample			MPN Index per 100 ml	95% Confidence limits	
	10 ml	1 ml	0.1 ml		Low	High
A1	4	4	0	34	12	93
A2	5	3	0	79	25	190
B1	5	5	3	920	300	3200
B2	5	4	3	350	120	1000
C1	5	5	3	920	300	3200
C2	5	5	2	540	180	1400

All the positive results of each lactose broth tube presented in this table have revealed growth of green metallic

Correlation between water quality parameters, fish density and the microbial density among the sampling sites

Correlation coefficients between water parameters (both physicochemical and microbiological), fish density and total isolated bacteria among all sampling sites were determined then presented in Table 11. Several negative strong and significant correlations were observed for the parameters: salinity, TDS, Secchi depth (water clarity meter) and pH with the total number of bacterial isolates (Wilcoxon, $P < 0.05$). They are plotted in separate graphs (Figure 7), except for the TDS that was exempted from these graphs due to its strong autocorrelation with salinity. Temperature, water flow, (TCF) and (FC) concentrations and fish density were all positively correlated to the total number of bacterial isolates, but the correlations were non-significant (Wilcoxon, $P > 0.05$). The (DO) revealed a non-significant negative moderate correlation (Wilcoxon, $P > 0.05$). Fish density showed a non-significant and moderate positive correlation with the total bacterial counts (Wilcoxon, $P > 0.05$).

Table 11. Correlation coefficients between water quality parameters, fish density, and the total number of isolated bacteria.

Parameter	[p] Total number of bacterial isolates rho value	Correlation strength	P-value
Temperature	0.1232082	Weak	0.8161
DO [g]	-0.6982533	Moderate	0.1228
***Salinity** [a, w, d]	-0.9411239	Strong	0.005098
***TDS** [a, w, d]	-0.9411239	Strong	0.005098
***** **pH** [w]	-0.8804063	Strong	0.0206
***Secchi depth** [g, d]	-0.8196886	Strong	0.04584
Water flow	0.6350006	Moderate	0.1755
Fish density	0.7700514	Moderate	0.07324
Total coliforms [c]	0.6253054	Moderate	0.1843
Fecal coliforms [c]	0.7084473	Moderate	0.1151

Legend: [a] (rho= 0.99, p-value = 0.003), [c] (rho = 0.85, p-value = 0.03), [w, g] (rho = 0.94, p-value = 0.01667), [d] rho = 0.89, p-value = 0.03333); [p] (total bacterial isolates among all sampling sites); * parameters that show significant difference and strong negative correlation with the bacterial isolation numbers.

4. Discussion

4.1 Physicochemical parameters

The results indicated fluctuations in water temperature within the three sampling sites with the lowest temperature at site B due to the highest freshwater discharge from the estuary towards the cages, which frequently carried the metabolic wastes of the fish away from the cages, in addition to the seawater fluxes during high tide that led to moderate water quality at this site and lower temperature compared to other study sites. This observation is similar to the temperature variation observed in another study in Setiu Wetland, they attributed it to the time of day of sampling, and on the tide (Suratman et al. 2016).

On the contrary, site C2 showed the highest temperature and lowest salinity, TDS, and pH compared with C1. This site had the highest fish density with potentially high presence of fish feed that could lead to: (i) high respiration rate due to the fish metabolic needs linked to the digestion of food; and (ii) increase fish excreta (ammonia) inputs, in addition to the effect of low water currents at site C1 and site C2. Consequently, that led to increased temperature and lowered pH, besides the low water flow that was probably insufficient to wash away the metabolic wastes. This is in agreement with other studies conducted at Setiu Wetlands,

where they correlated low pH with low DO concentration in the water column. They suggested that the excess fish food and faces discharged from aquaculture are a major factor contributing to the increased organic decomposition rate, which is responsible for decreasing DO and pH in the water column. (Suratman et al. 2014; 2016).

Noteworthy, when comparing site A2 with site C1 that both had the same fish density (24 fish/m^3) and cage size, and that both received the lowest water inflow, A2 showed significantly higher mean DO, salinity, TDS and pH ($P < 0.05$, except for the temperature that was similar at both sites. This could be due to (i) widely buffered condition of site A from the seawater currents during high tide, conversely to site C that was subjected more to freshwater fluxes from the estuary, which was markedly discerned with the decreased salinity, TDS, DO, pH and water transparency at site C; (ii) different oxygen requirement of different fish species (grouper at site A2 versus seabass at site C1); and (iii) possibly higher organic matter decomposition at site C1. Another point of interest was the sites B2 and C2 that had the same fish species but slightly different fish density (29.23 and 32.68 fish/m^3, respectively). We noted the significantly higher temperature, reduced salinity, TDS, and pH at site C2 contrary to site B2, ascribed to higher water flow at site B2, which offset the effects of high fish density at site B2. The DO concentration was not different between these sites, but if compared with sites A2 and C1 that held different fish species at the same density, it seemed to suggest that the fish species at the latter sites had different rates of respiration. A similar observation was reported by Suratman et al. (2016), where the DO concentration at the same Wetland area was also found to be low due to the respiration activities of the fish inside the cage area.

Although water transparency (Secchi depth) showed no significant difference between the study sites, site C showed the lowest water transparency due to its vicinity from the estuary location (Fig. 1 (A)).

The findings showed that some of the water quality parameters such as the salinity had exceeded the optimal range (15–25) ppt for the grouper at site A (Noor et al., 2018) and pH levels were less than the optimal range (7.5–8.5) for the seabass (Anil et al., 2010) at site C2, (see Table 2), which means the grouper fish in this study were under salinity stress, and the seabass species at site C2 exposed to non-optimal pH. Thus, we advise the farmers to culture grouper species within the recommended salinity range mentioned by Noor et al., 2018 and seabass under pH levels of 7.5–8.5 for better health and growth of the cultured fish, and to sustain the economic profits.

Although the physicochemical water parameters were not different between the cages of low and high fish densities, except the C site, we specified the significant water parameters that correlated with the total number of bacterial isolations in cultured fish. These parameters were mainly the salinity, TDS, pH, and water transparency that were observed to have strong negative associations with the presence of total bacterial isolations in all the sampling sites.

In general, there was a significant reduction in the mean values of DO, TDS, salinity, and pH from sites A to B towards site C (see Table 2). The water quality was mostly influenced by the environment (*e.g.*, river flow, seawater surges), but not much by fish density, except at site C where the water mixing and the dilution of fish waste were poor (Fig. 1).

4.2 Coliform counts

The results indicated high total coliforms (TCF) at site C1, and the lowest at site A, while fecal thermotolerant coliforms (FC) were also the lowest at site A but were different among the other sites. The reasons behind high coliform concentration at C sites may be due to the direct discharge of wastewater from septic tank, industrial wastewater to the Setiu river and due to the vicinity of C sites from the river mouth (Fig. 1). Contrary to A sites that are mostly washed by sea water currents during the high tides (Fig. 1 and Table 2). According to Bartram and Rees (2000), the decay rate (die-off) of Enterococci and fecal coliforms (e.g., *E. coli*) is higher at high salinity, under high solar radiation exposure (UV effects) and in the absence of rainfall and vice versa, except for Enterococci that were not affected by the sunlight.

The low water flow rate and high-water clarity at site A likely facilitated the penetration of solar light into the water column, in addition to the high salinity and pH during solar exposure were considered as exogenous factors that play an important role in accelerated microbial decay rate (Nelson et al., 2018) which indicates the reasons why the coliform concentrations are low at A sites Tables 2 and 5. Similar results were found in Selangor river where they attributed the presence of coliform contamination due to the existence of many sewage discharges and industrial effluents along the river (Faridah et al., 2015). Besides, polluted river water could have brought coliforms from the surrounding residential area to the estuary, especially in their attached form to soil particles. This form is known to protect microorganisms from the sunlight effects in turbid water. Hence, the variation of (TCF) and (FC) concentrations at sites B and C are associated with hydrodynamics driven by water flow, salinity changes, and the sedimentation rate of the suspended or dissolved particles (water transparency).

The total coliform and fecal data counts have indicated the farming area pollution with animal and human feces; however, they didn't show any significant correlation with the total numbers of bacterial isolation (Table 11).

4.2 Bacterial isolates

The bacteriology results, at first, the observed differences in the total number of bacterial isolation from site A compared to sites B1, C1, and C2 (Fig. 2) could suggest that the fish stocking density influenced the microbial isolation, which was the lowest at the site A (Table 1) compared to the fish densities at the other sites. But, if we look at the dramatic change in water properties for these sites as if we move from site A towards C where the estuary is located, and also to the correlation coefficient between fish density and the total number of microbial isolates (Table 11), this could imply that fish density has no direct effect on the total number of bacterial isolates.

In addition, although the numbers of isolated species seemed to be higher in sites B and C, the results showed no difference between all the isolated species from low and high fish densities; therefore, our first hypothesis is rejected. In comparison with other studies, Chakraborty et al. (2010) postulated that 50 fish/m^3 is optimum stocking density while 75 to 100 fish/m^3 is considered high to Nile tilapia species because within those densities a rise in plasma cortisol concentrations was evident due to social stress between fish individuals as well as the survival rate was reduced by 60%. Farhaduzzaman et al. (2020) have

recommended that the optimum density should not exceed 35 to 43 fish/m^3. This suggests that our selected fish stocking densities were within normal ranges in this study (Table 1), which is why the fish density showed no effects on the pathogen numbers, the species numbers, and overall bacterial isolations. Nevertheless, the isolated bacterial species were variable among sampling sites, it could be due to the differences in water parameters among the sampling sites (Fig. 3, Tables 2 and 8). Similarly, other studies have reported that physico-chemical parameters showed their importance in influencing the occurrence and the density of bacterial populations (Gorlach et *al*., 2013; Amal et al., 2015; Nurul Izzatul et al., 2016).

The significant difference in total pathogens isolation between the sites A1 and A2 with the site C2, (Fig. 4) is attributed to different factors; (i) if we closely observe the fish densities at sites A1 and A2, and compared them with densities of sites C1 and C2 respectively (see Table 1); (ii) check the water quality between the A and C sites (see Table 2), we spot both A2 and C1 have the same density; A2 differed from C2 in the number of pathogen isolation but not C1 from C2 (Fig. 4). The main points here are the differences in the fish species and water quality parameters (the concept was discussed previously in the water quality part).

The higher number of microbes isolates at site C2 can be explained by several factors like excess feed inputs, an increase in fish respiration, metabolic waste accumulation, and temperature, plus the slower water inflow into the cage surroundings, the low salinity, and the water clarity. Moreover, the non-optimal pH maybe stresses the cultured fish, and make them more susceptible to bacterial infections (see Table 2 and Fig. 4).

Generally, among all sampling sites we observed that the salinity, TDS, pH, and water transparency were strongly negatively correlated with the occurrence of total bacterial isolates (see Figs. 2 & 5, and Tables 2 & 11), while neither the fish density nor the coliform concentrations did show a significant correlation with total bacterial isolates among all sites (see Table 11).

Despite bacteria have recovery strategies to repair sub-fatal injuries after sunlight damage, there is evidence that sunlight exposure is known to cause immediate damage to bacterial DNA, their cell membranes, cytoplasmic proteins, scavenging enzymes, *e.g.*, direct photolysis of catalase, in addition to other factors such as higher salinity and pH during solar irradiation (Nelson et al., 2018). They likely influenced the microbial density and composition in the environment surrounding site A.

As we observed, that water characteristics have favored the cultured seabass to harbor more pathogens at sites B and C, such pathogens may be also the result of the diseased or carrier fish. More specifically, a non-optimal pH level was noticed at site C2 with a potential of high microbial load surrounding the cages (Table 9). On the other hand, although grouper species were stocked in the lowest densities (*i.e.*, less crowding contact between individuals) (see Table 1) and potentially less microbial load in the surrounding water compared to other study sites (Table 9), the non-optimal salinity levels are considered a stressful condition to these species (Noor et al., 2018), that led to some bacterial infections.

Additionally, the mucosal surfaces of the fish skin and gills are colonized by commensal microorganisms that act as a primary defense mechanism to protect the fish individuals from the pathogenic challenges, but any alteration in their abundance leads to increased susceptibility to opportunistic pathogens. For example,

Musharrafieh et al. (2014) revealed that fish transport and handling led to acute stress that altered the commensal bacteria of the rainbow trout skin; likewise, Zhang et al. (2018) reported that parasite infection led to a decreased abundance of skin commensals in a teleost fish experiment.

A broad range of bacterial taxa was isolated from cultured seabass and grouper that showed no clinical sign of disease like in other published studies, which were mostly Gram-positive (Marcel et al., 2013, Scarano et al., 2014; Nurul Izzatul et al., 2016) except for one isolate which was from congested kidneys.

The dominant bacteria in our study were *Staphylococcus warneri* and *Staphylococcus sciuri*, which were recovered from the skin and gills of the fish samples. In general, these species are found as commensal microbiomes in the fish (Musharrafieh et al., 2014; Beims et al., 2016), including *Staphylococcus vitulinus*, but it also mentioned as pathogens with previous reports in a different study on the fish (Beims et al., 2016).

In this study, we additionally isolated *Staphylococcus pseudintermedius*, a commensal bacterium in the dog's skin. Its presence in sites A and B suggests either that the water surrounding the cage culture or the net-transfer equipment was contaminated with infected dogs or their mucus.

The detection of *Proteus mirabilis*, *Klebsiella oxytoca*, and *Enterococcus* species in fish mainly occurred at sites B and C, which may originate from the contaminated water surrounding the cage culture with the excretion of animals and human feces, (Table 8).

The members of the *Bacillus cereus* group were *B. cereus*, *B. thuringiensis*, and *B. mycoides*, they are ubiquitous in nature, soil, and foods; they are spore-forming bacteria, also have a saprophytic life cycle and can germinate in the soil (Ehling-Schulz et al., 2018).

All our isolates of the *Bacillus cereus* group showed beta hemolytic activity, yet, the pathogenicity of these species is variable: from an opportunistic pathogen, *B. cereus*, entomopathogens, *B. thuringiensis* (Ehling-Schulz et al., 2018), while strains of *B. mycoides* have not been reported as pathogenic yet.

The species *Micrococcus lutes*, *Pseudomonas aeruginosa*, and *Aeromonas hydrophila* found in this study are similar to other findings, these species had also been isolated and identified as pathogenic strains from freshwater fish in Malaysia by Marcel et al. (2013).

Leuconostoc pseudomesenteroides found in this study had also been previously isolated from fecal samples of Tibetan yaks and regarded as a potential bacteriostatic agent against pathogens (Wang et al., 2018).

In this study, we isolated several other pathogens, which consisted of *Streptococcus agalactiae* and *Lactococcus garviae*, they are reported as major etiologic agents of fish diseases in aquaculture (Mishra et al., 2018; Pękala-Safińska, 2018); *Acinetobacter radioresistens* and *Aerococcus viridans* are widely dispersed in nature, aquatic environment, sewage, and soil, it causes different diseases to animals and humans (Al Atrouni et al., 2016); The isolated species *Sphingomonas paucimobilis* and *Stenotrophomonas maltophilia* are considered among the most frequent pathogens in fish (Pękala-Safińska, 2018); *Kocuria kristinae* is acknowledged as skin commensal in mammals but is also found in various environmental

niches (Pękala-Safińska, 2018). Therefore, we assumed that such bacteria might be derived from the polluted water that contaminating the site B; *Rhizobium radiobacter* is a saprophytic bacterium found in soil and plants, its presence in our isolates is attributed to the contamination of water with these bacteria. It's reported as an opportunistic pathogen to immunocompromised humans (Stamou et al., 2018).

Finally, *Vibrio* species are widespread in the estuarine and coastal marine environments and was reported in other studies to cause economically important diseases in the cultured fish farms in different countries (Ina-Salwany et al., 2019).

Since the mechanism of α and β hemolysins is based on a receptor-mediated interaction with the cytoplasmic membranes of target cells (Kong et al., 2016); we speculated the reason why the majority of the isolates lacked γ-hemolysin expression is that the phospholipids forming the membrane surfaces of the tested erythrocytes were probably more sensitive to the produced α toxins or the enzymatic properties of the produced hemolysin may vary between the isolated strains in this study. Therefore, we have selected the pathogenic isolates with β-hemolysis to be tested for antibiotic susceptibility.

Although there was no evidence of tissue damage (excluding the congested kidneys sample) of the fish organs due to pathogens in this study, the presence of alpha strains does not necessarily cause serious disease problems in fish aquaculture, in comparison with the beta strains that impose a higher risk of pathogenicity (this was illustrated by complete lysis of erythrocytes on blood agar). Nevertheless, based on the frequency of occurrence of each pathogen per sampling site (Table 8), we consider the fish farm healthy and not vulnerable to disease outbreaks.

As the majority of bacterial isolates lacked γ-hemolysin expression, which was our measure to determine the commensal strains to investigate the fish health, based on the frequency percentages of each pathogen per sampling site, we don't consider that an alarming sign for the health status on this farm.

4.3 Antibiotic susceptibility

In the present study, the observed resistance of strains of the *Bacillus cereus* group against the β-lactam antibiotics (P and AM) is associated with the production of a potent broad-spectrum of β-lactamase that inhibiting the effects of these drugs as commonly reported by CLSI (2015). The variation in the levels of antibiotic resistance found in this group might be due to the geographical location with respect to the potential of antibiotics exposure or to the mechanisms of resistance that may vary between the isolated strains e.g., antibiotic efflux systems for tetracycline and chloramphenicol Hassan et al. (2017). Thus, we attributed such differences to resistance mechanisms among tested strains and variability of antibiotic exposure in the farming area.

The high efficacy exhibited by fluoroquinolone drug (CP) is because its mode of action is DNA gyrase inhibition (Dincer et al., 2017), these enzymes are necessary for DNA replication in the host cell and are not affected by plasmid or enzyme-mediated resistance unless chromosomal mutations occur LeBel (1988). In contrast, aminoglycosides target the subunit 30S of the ribosome in prokaryotes, which is mainly responsible for protein and cell membrane synthesis (Sultan et al. 2018).

In the current study, the lowest sensitivity was observed towards (SM) among the other tested aminoglycosides (GM and KM), it's probably either due to the application on this farm or to the high exposure to such drug that loaded with water fluxes into this farm, which led to reduced sensitivity and increased resistance patterns.

The observed resistance of *P. aeruginosa* against (KM), might be it is originated from a hospital waste driven in the polluted water, while (GM) resistance by R. *radiobacter* could be through the fluxes of such drug in polluted water. Similarly, other studies stated that the highest antibiotics quantities are found in estuaries which are originated from domestic and hospital wastes as well as the resultants of disease treatment in aquacultures (Dincer et al. 2017).

The susceptibility profiles of the pathogens isolated from low fish density were significantly lower (i.e., low median values of inhibition zones were obtained) compared to the isolates from high fish density. However, such variations could be due to (i) water flow rates were different among samplings sites which made some unequal differences in antibiotic discharge among the sites (Dincer et al. 2017); (ii) the antibiotic (SM) maybe has been applied to this farm because it had the lowest sensitivity rates compared to other drugs in this study; (iii) either bacterial mutations or the horizontal gene transfer with other microorganisms in the natural environment (Dincer et al. 2017); and lastly (iv) might the tested sample numbers were not sufficient and equal among the tested drugs between the two fish densities.

5. Conclusions

Our findings in the present study indicated that fish density has no effects on the pathogen numbers, species numbers, and overall bacterial isolations. This suggests that the fish stocking densities were within the optimum in this study.

In this survey, we isolated several bacterial species from the fish, which may be associated with the nature of the environment and water quality surrounding the net cages. However, the environmental variables are far more easily exposed to fluctuations, which makes it necessary for further study to investigate such relationships during the wet season. Furthermore, a comprehensive estimation of the health risk of this farm is periodically required by using advanced molecular techniques (*e.g.*, examine the virulence genes of pathogenic isolates) to address the problem of infected fish with no clinical signs.

Declarations

Financial disclosure statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Animal rights statement

No permission of research and animal ethics was necessary because the fish were purchased from a commercially fish farm, therefore no endangered species were collected for the study.

Credit authorship contribution statement

Fieldwork, sample collection, all laboratory work, and data analysis were carried out by Mohammed M.Tawfeeq. Mohd Effendy, Musa Najiah, and Isabelle G. are equally contributed to the manuscript editing, pertinent support, and valuable comments. All authors contributed to the article and approved the submitted version.

Declaration of competing interest

We have no competing interests.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Abbreviations

DO: Dissolved Oxygen

AM: Ampicillin

CL: Chloramphenicol

CLSI: Clinical and Laboratory Standards Institute

CP: Ciprofloxacin

EMB: Eosin Methylene Blue

FC: thermotolerant fecal coliforms

GM: Gentamicin

GPS: Geographic Positioning System

KM: Kanamycin

MMR: The Maximum: Minimum Ratio

MPN: Most Probable Number

NCCLS: National Committee for Clinical Laboratory Standards

P: Penicillin

SM: Streptomycin

TC: Tetracycline

TCBS: Thiosulphate-Citrate Bile Salts Sucrose

TCF: Total coliform

TDS: Total Dissolved Solids

WHO: World Health Organization

ZMA: Zobell Marine Agar

α: Alpha hemolysin

β: Beta hemolysin

γ: Gamma hemolysin

References

Al Atrouni, Ahmad, Marie-Laure Joly-Guillou, Monzer Hamze, and Marie Kempf. "Reservoirs of Non-*Baumannii acinetobacter* Species." *Frontiers in Microbiology* 7 (2016): 49. https://doi.org/10.3389/fmicb.2016.00049.

Amal, Mohammad Noor Azmai, Mohd Zamri Saad, Abdullah Siti Zahrah, and Abd Rashid Zulkafli. "Water Quality Influences The Presence of *Streptococcus agalactiae* in Cage Cultured Red Hybrid Tilapia, *Oreochromis niloticus* × *Oreochromis mossambicus*." *Aquaculture Research* 46, no. 2 (2015): 313–23. https://doi.org/10.1111/are.12180.

Anil, M K and Santhosh, B and Jasmine, S and Saleela, K N and George, Rani Mary and Kingsly, H

Jose and Unnikrishnan, C and Rao, A Hanumantha and Rao, G Syda. “Growth Performance of The Seabass *Lates calcarifer* (Blotch) in Sea Cage at Vizhinjam Bay Along the South-West Coast of India”. *Indian Journal of Fisheries*, 57 (4), (2010): pp. 65-69.

Assefa, Ayalew, and Fufa Abunna. “Maintenance of Fish Health in Aquaculture: Review of Epidemiological Approaches for Prevention and Control of Infectious Disease of Fish.” Edited by William Ravis. *Veterinary Medicine International* 2018 (2018): 5432497. https://doi.org/10.1155/2018/5432497.

Beims, H, A Overmann, M Fulde, M Steinert, and S Bergmann. “Isolation of *Staphylococcus sciuri* from Horse Skin Infection.” *Open Veterinary Journal* 6, no. 3 (2016): 242–46. https://doi.org/10.4314/ovj.v6i3.14.

Bumadian, Mohamed. “Detection and Enumeration of Coliform Bacteria in Drinking Water at Hospital of Benghazi, Libya”. *Journal of Experimental Biology and Agricultural Sciences*, 1(6):436-440., October 2013.

Chakraborty, Suman Bhusan, Debasis Mazumdar, and Samir Banerjee. “Determination of Ideal Stocking Density for Cage Culture of Monosex Nile Tilapia (*Oreochromis niloticus*) in India.” *Proceedings of the Zoological Society* 63, no. 1 (2010): 53–59. https://doi.org/10.1007/s12595-010-0007-3.

Chitmanat, Chanagun, Phimphakan Lebel, Niwooti Whangchai, Jongkon Promya, and Louis Lebel. “Tilapia Diseases and Management in River-Based Cage Aquaculture in Northern Thailand.” *Journal of Applied Aquaculture* 28, no. 1 (2016): 9–16. https://doi.org/10.1080/10454438.2015.1104950.

Chow, AW, MJ Gribble, and KH Bartlett. “Characterization of the Hemolytic Activity of *Staphylococcus aureus* Strains Associated with Toxic Shock Syndrome.” *Journal of Clinical Microbiology* 17, no. 3 (1983): 524—528. https://doi.org/10.1128/jcm.17.3.524-528.1983.

Clinical and Laboratory Standards Institute (CLSI). Methods for Antimicrobial Dilution and Disk

Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. 3rd ed. (2015): CLSI

guideline M45. Wayne, PA.

Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial

Susceptibility Testing. 27th ed. CLSI supplement M100 (ISBN 1-56238-804-5 [Print]; ISBN 1.

(2017): 56238-805-3 [Electronic]). Clinical and Laboratory Standards Institute, 950 West Valley

Road, Suite 2500, Wayne, Pennsylvania 19087 USA.

Conte, F.S. “Stress and the Welfare of Cultured Fish.” *Applied Animal Behaviour Science* 86, no. 3 (2004): 205–23. https://doi.org/10.1016/j.applanim.2004.02.003.

Das, A., B. K. Behera, S. Acharya, P. Paria, H. J. Chakraborty, P. K. Parida, and B. K. Das. “Genetic Diversity and Multiple Antibiotic Resistance Index Study of Bacterial Pathogen, *Klebsiella pneumoniae* Strains

Isolated from Diseased Indian Major Carps." *Folia Microbiologica* 64, no. 6 (2019): 875–87. https://doi.org/10.1007/s12223-019-00701-7.

Di, Jun, Shuhuan Zhang, Jun Huang, Hao Du, Yong Zhou, Qiong Zhou, and Qiwei Wei. "Isolation and Identification of Pathogens Causing Haemorrhagic Septicaemia in Cultured Chinese Sturgeon (*Acipenser sinensis*)." *Aquaculture Research* 49, no. 11 (2018): 3624–33. https://doi.org/10.1111/are.13830.

Duarte, Letícia N., Francisco J. R. C. Coelho, Vanessa Oliveira, Daniel F. R. Cleary, Patrícia Martins, and Newton C. M. Gomes. "Characterization of Bacterioplankton Communities from a Hatchery Recirculating Aquaculture System (RAS) for Juvenile Sole (*Solea senegalensis*) Production." *PLOS ONE* 14, no. 1 (2019): 1–16. https://doi.org/10.1371/journal.pone.0211209.

Dincer, S., & Yigittekin, E. S. "Spreading of Antibiotic Resistance with Wastewater. In Biological

Wastewater Treatment and Resource Recovery." *Intech Open Limited*. (2017). doi:

10.5772/66188.

Ehling-Schulz, Monika, Didier Lereclus, and Theresa M Koehler. "The *Bacillus cereus* Group: *Bacillus* Species with Pathogenic Potential." *Microbiology Spectrum* 7, no. 3 (2019): 10.1128/microbiolspec.GPP3-0032–2018. https://doi.org/10.1128/microbiolspec.GPP3-0032-2018.

Farhaduzzaman, A.M, Md Hanif, Suzan Khan, Mahadi Osman, Md Neamul, Hasan Shovon, Md Rahman, and Shahida Ahmed. "Perfect Stocking Density Ensures Best Production and Economic Returns in Floating Cage Aquaculture System." *Journal of Aquaculture Research and Development* 11 (2020): 607. https://doi.org/10.35248/2155-9546.20.10.607.

G, Marcel, Y M, Siti A, and Benjamin Emikpe. "Water Condition and Identification of Potential Pathogenic Bacteria from Red Tilapia Reared in Cage-Cultured System in Two Different Water Bodies in Malaysia." *African Journal of Microbiology Research* 7 (2013): 5330–37. https://doi.org/10.5897/AJMR12.1468.

Gorlach-Lira, K., Pacheco, C., Carvalho, L.C.T., MeloJúnior, H.N., Crispim, M.C., "The influence of fish Culture in Floating Net Cages on Microbial Indicators of Water Quality." *Brazilian Journal of Biology* [online]. (2013): v. 73, n. 3, pp. 457-463. https://doi.org/10.1590/S1519-69842013000300001.

Hassan, Karl A., Annette Fagerlund, Liam D. H. Elbourne, Aniko Vörös, Jasmin K. Kroeger, Roger Simm, Nicolas J. Tourasse, et al. "The Putative Drug Efflux Systems of the *Bacillus cereus* Group." *PLOS ONE* 12, no. 5 (2017): 1–25. https://doi.org/10.1371/journal.pone.0176188.

Ina-Salwany, M. Y., Nurhidayu Al-saari, Aslah Mohamad, Fathin-Amirah Mursidi, Aslizah Mohd-Aris, M. N. A. Amal, Hisae Kasai, Sayaka Mino, Tomoo Sawabe, and M. Zamri-Saad. "Vibriosis in Fish: A Review on Disease Development and Prevention." *Journal of Aquatic Animal Health* 31, no. 1 (2019): 3–22. https://doi.org/10.1002/aah.10045.

Jackie Reynolds, , Kirby-Bauer (Antibiotic Sensitivity), Richland College, (2019). Available at:

(https://bio.libretexts.org/Ancillary_Materials/Laboratory_Experiments/Microbiology_Labs_I/093A_Kirby-Bauer_(Antibiotic_Sensitivity). (Accessed on July 2019).

Jamie Bartram and Gareth Rees, (2000). Monitoring Bathing Waters - A Practical Guide to the Design and Implementation of Assessments and Monitoring Programmes: ISBN 0-419-24390-1. Available at: (www.who.int/water_sanitation_health/bathing/monbathwat.pdf). (Accessed: 1st May 2018).

Ismail, Nurul Izzatul Aliya, Mohammad Noor Azmai Amal, Shamarina Shohaimi, Mohd Zamri Saad, and Siti Zahrah Abdullah. "Associations of Water Quality and Bacteria Presence in Cage Cultured Red Hybrid Tilapia, *Oreochromis niloticus* × *O. mossambicus*." *Aquaculture Reports* 4 (2016): 57–65. https://doi.org/10.1016/j.aqrep.2016.06.004.

Kong, Cin, Hui-min Neoh, and Sheila Nathan. "Targeting *Staphylococcus aureus* Toxins: A Potential Form of Anti-Virulence Therapy." *Toxins* 8, no. 3 (2016): 72. https://doi.org/10.3390/toxins8030072.

LeBel, Marc. "Ciprofloxacin: Chemistry, Mechanism of Action, Resistance, Antimicrobial Spectrum, Pharmacokinetics, Clinical Trials, and Adverse Reactions." Pharmacotherapy: *The Journal of Human Pharmacology and Drug Therapy* 8, no. 1 (1988): 3–30. https://doi.org/10.1002/j.1875-9114.1988.tb04058.x.

López Nadal, Adrià, David Peggs, Geert F. Wiegertjes, and Sylvia Brugman. "Exposure to Antibiotics Affects Saponin Immersion-Induced Immune Stimulation and Shift in Microbial Composition in Zebrafish Larvae." *Frontiers in Microbiology* 9 (2018): 2588. https://doi.org/10.3389/fmicb.2018.02588.

Miller, Ron A., Heather Harbottle, Frank Møller Aarestrup, Stefan Schwarz, Jianzhong Shen, and Lina Cavaco. "Antimicrobial Drug Resistance in Fish Pathogens." *Microbiology Spectrum* 6, no. 1 (2018): 6.1.07. https://doi.org/10.1128/microbiolspec.ARBA-0017-2017.

Mishra, Anshuman, Gyu-Hwi Nam, Jeong-An Gim, Hee-Eun Lee, Ara Jo, and Heui-Soo Kim. "Current Challenges of *Streptococcus* Infection and Effective Molecular, Cellular, and Environmental Control Methods in Aquaculture." *Molecules and Cells* 41, no. 6 (2018): 495–505. https://doi.org/10.14348/molcells.2018.2154.

Mishra, Meerambika, Ananta P. Arukha, Amiya K. Patel, Niranjan Behera, Tapan K. Mohanta, and Dhananjay Yadav. “Multi-Drug Resistant Coliform: Water Sanitary Standards and Health Hazards.” *Frontiers in Pharmacology* 9 (2018): 311. https://doi.org/10.3389/fphar.2018.00311.

Musharrafieh, Rami, Luca Tacchi, Joshua Trujeque, Scott LaPatra, and Irene Salinas. “*Staphylococcus warneri,* a Resident Skin Commensal of Rainbow Trout (*Oncorhynchus mykiss*) with Pathobiont Characteristics.” *Veterinary Microbiology* 169, no. 1–2 (2014): 80–88. https://doi.org/10.1016/j.vetmic.2013.12.012.

Nagasawa, K. and E. R. Cruz-Lacierda (eds.). “Diseases of Cultured Groupers.” Southeast Asian

Fisheries Development Center, Aquaculture Department, Iloilo, Philippines. 2004: 81 p.

NCCLS., Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for

Bacteria Isolated from Animals; Approved Standard—Second Edition. NCCLS document M31

A2 (ISBN 1-56238), (2002).

Nelson, Kara L, Alexandria B Boehm, Robert J Davies-Colley, Michael C Dodd, Tamar Kohn, Karl G Linden, Yuanyuan Liu, et al. “Sunlight-Mediated Inactivation of Health-Relevant Microorganisms in Water: A Review of Mechanisms and Modeling Approaches.” *Environmental Science. Processes & Impacts* 20, no. 8 (August 16, 2018): 1089–1122. https://doi.org/10.1039/c8em00047f.

Noor, N. M., S. K. Das, Z. C. Cob, and M. A. Ghaffar. “Effects of Salinities and Diets on Growth of Juvenile Hybrid Grouper, *Epinephelus fuscoguttatus and E. lanceolattus*.” *Turkish Journal of Fisheries and Aquatic Sciences* 18 (2018): 1045–51.

Othman, Faridah, Md.SadekUddin Chowdhury, and Nobumitsu Sakai. “Assessment of Microorganism Pollution of Selangor River, Malaysia.” International Conference on Agricultural, Ecological and Medical Sciences (AEMS-2015), (2015). https://doi.org/10.15242/IJAAEE.C0215147.

Pękala-Safińska, Agnieszka. “Contemporary Threats of Bacterial Infections in Freshwater Fish.” *Journal of Veterinary Research* 62, no. 3 (2018): 261–67. https://doi.org/10.2478/jvetres-2018-0037.

Pincus, David, (2014). “Microbial Identification Using The Biomerieux VITEK ® 2 System.”

J. Encyclopedia of Rapid Microbiological Methods. (2014). 2.

Public Health England, (2017), Inoculation of Culture Media for Bacteriology. UK standards for

microbiology investigations (SMI). Q 5 Issue 2. Available

at:(https://www.gov.uk/government/publications/smi-q-5-inoculation-of-culture media-for-bacteriology). (Accessed: 20th June 2018).

Scarano, Christian, Carlo Spanu, Graziella Ziino, Francesca Pedonese, Alessandra Dalmasso, Vincenzo Spanu, Salvatore Virdis, and Enrico De Santis. “Antibiotic Resistance of *Vibrio* Species Isolated from *Sparus aurata* Reared in Italian Mariculture.” *The New Microbiologica: Official Journal of the Italian Society for Medical Virology* (SIVIM) 37 (June 2014): 329–37.

Stamou, Aikaterini, Charalampos Pavlopoulos, Stefanos Roumeliotis, Efthymios Samoladas, Ippokratis Xatzokos, and Konstantina Kontopoulou. “Nonunion Humerous Fracture Infection Caused by *Rhizobium radiobacter* in a 24-Year-Old Healthy Patient: A Rare Case Report.” *Case Reports in Infectious Diseases* 2018 (2018): 8627165–8627165. https://doi.org/10.1155/2018/8627165.

Sultan, Insha, Safikur Rahman, Arif Tasleem Jan, Mohammad Tahir Siddiqui, Aftab Hossain Mondal, and Qazi Mohd Rizwanul Haq. “Antibiotics, Resistome and Resistance Mechanisms: A Bacterial Perspective.” *Frontiers in Microbiology* 9 (2018): 2066. https://doi.org/10.3389/fmicb.2018.02066.

Suratman, Suhaimi, A Hussein, Norhayati Mohd Tahir, Mohd Talib Latif, Roslanzairi Mostapa, and K Weston. “Seasonal and Spatial Variability of Selected Surface Water Quality Parameters in Setiu Wetland, Terengganu, Malaysia.” *Sains Malaysiana* 45 (2016): 551–58.

Wang, Wei, Jing Sun, Cenjie Liu, and Zhuang Xue. “Application of Immunostimulants in Aquaculture: Current Knowledge and Future Perspectives.” *Aquaculture Research* 48, no. 1 (2017): 1–23. https://doi.org/10.1111/are.13161.

Wang, Yanfeng, Liang Chi, Qinghua Liu, Yongshuang Xiao, Daoyuan Ma, Zhizhong Xiao, Shihong Xu, and Jun Li. “Effects of Stocking Density on the Growth and Immunity of Atlantic Salmon Salmo Salar Reared in Recirculating Aquaculture System (RAS).” *Journal of Oceanology and Limnology* 37, no. 1 (2019): 350–60. https://doi.org/10.1007/s00343-019-7350-7.

Wang, Yaping, Aoyun Li, Xiong Jiang, Hui Zhang, Khalid Mehmood, Lihong Zhang, Jinhuan Jiang, Muhammad Waqas, Mujahid Iqbal, and Jiakui Li. “Probiotic Potential of *Leuconostoc pseudomesenteroides* and *Lactobacillus* Strains Isolated from Yaks.” *Frontiers in Microbiology* 9 (2018): 2987. https://doi.org/10.3389/fmicb.2018.02987.

Wang, Yi-Wei, Jian Zhu, Xian-ping Ge, Sheng-Ming Sun, Yan-Li Su, Bing Li, Yi-Ran Hou, and Ming-Chun Ren. “Effects of Stocking Density on the Growth Performance, Digestive Enzyme Activities, Antioxidant Resistance, and Intestinal Microflora of Blunt Snout Bream (*Megalobrama amblycephala*) Juveniles.” *Aquaculture Research* 50, no. 1 (2019): 236–46. https://doi.org/10.1111/are.13889.

WHO:Multiple Tube Method for Thermotolerant (Faecal Coliform). Available at:

(https://www.who.int/water_sanitation_health/water-quality/small-community

management/2edvol3i.pdf?ua=1). (Accessed; 18th June 2018).

World Organisation for Animal Health (OIE), (2018). OIE Annual report on the use of antimicrobial

agents in animals. Available at: (http://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/AMR/Annual_ReportMR_3.pdf).

Zamri-Saad, M., Amal, M.N.A., Siti-Zahrah, A., Zulkafli, A.R., "Control and Prevention of *Streptococcosis* in Cultured Tilapia in Malaysia: a review." *Pertanika J. Trop. Agric Sci*. 37:(4), (2014):389–410.

Zhang, Xiaoting, Liguo Ding, Yongyao Yu, Weiguang Kong, Yaxing Yin, Zhenyu Huang, Xuezhen Zhang, and Zhen Xu. "The Change of Teleost Skin Commensal Microbiota Is Associated with Skin Mucosal Transcriptomic Responses during Parasitic Infection by *Ichthyophthirius multifillis*." *Frontiers in Immunology* 9 (2018): 2972. https://doi.org/10.3389/fimmu.2018.02972.

Figures

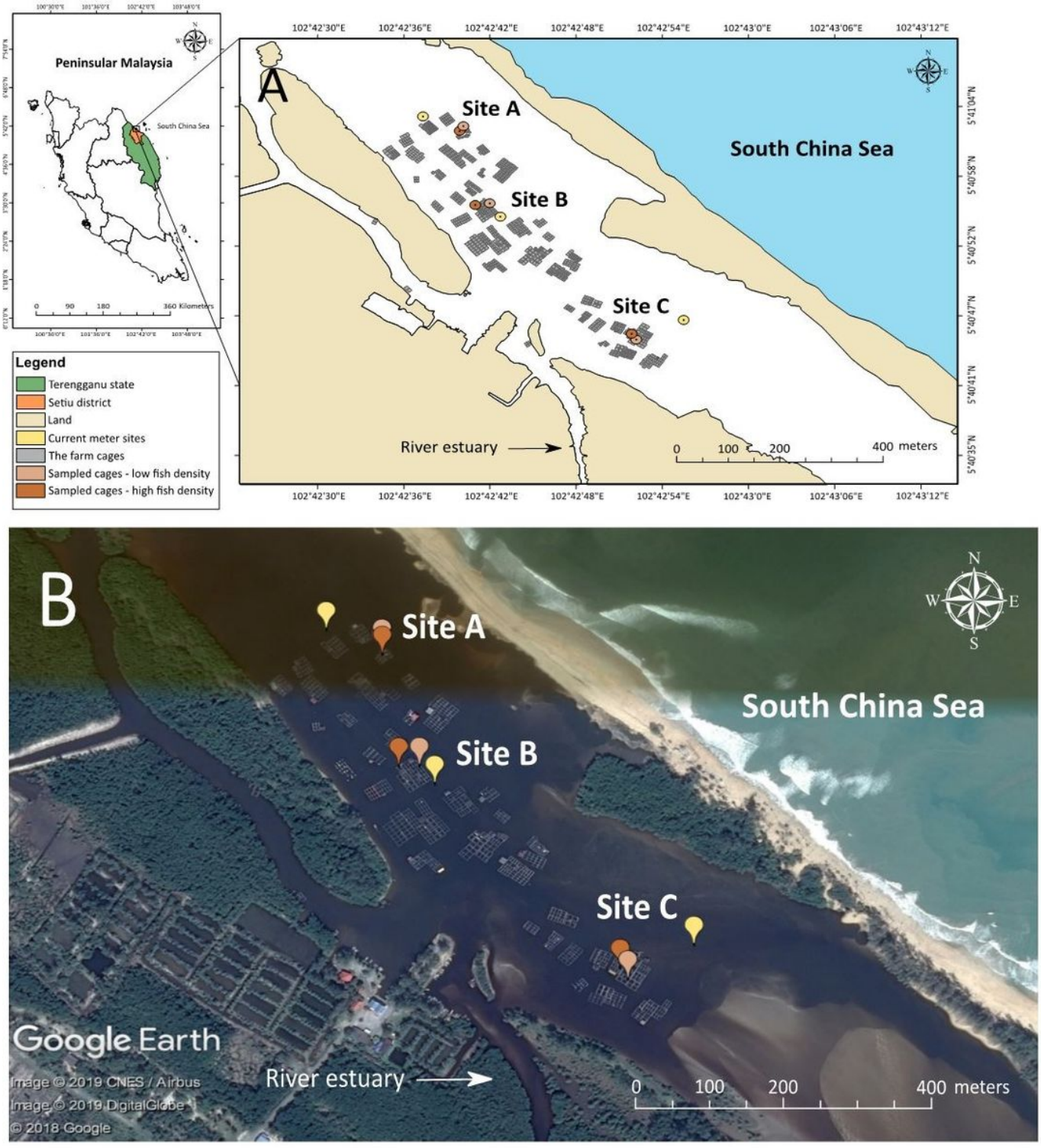

Figure 1

(A) Map of the study area showing the Fish sampling and the water flow measurements sites, Setiu wetlands, Terengganu, Malaysia. (Peninsular Malaysia map was downloaded from (www.diva-gis.org/gdata), and by using the software "ArcGIS" was created figure (A) a digitized copy of a picture was obtained from Google earth). (B) Picture of the study area obtained from satellite imaging using Google earth software.

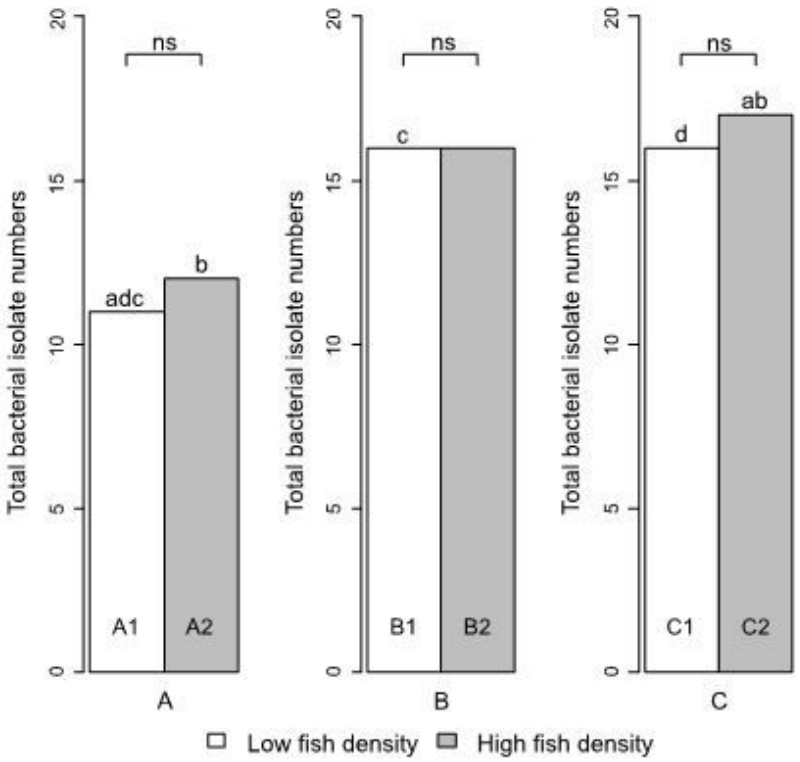

Figure 2

Total numbers of bacteria isolated from fish stocke in low and high densities at the different sampling sites. (significance levels: a = 0.008, c = 0.025, b=0.03, d= 0.059, ns = non-significant)

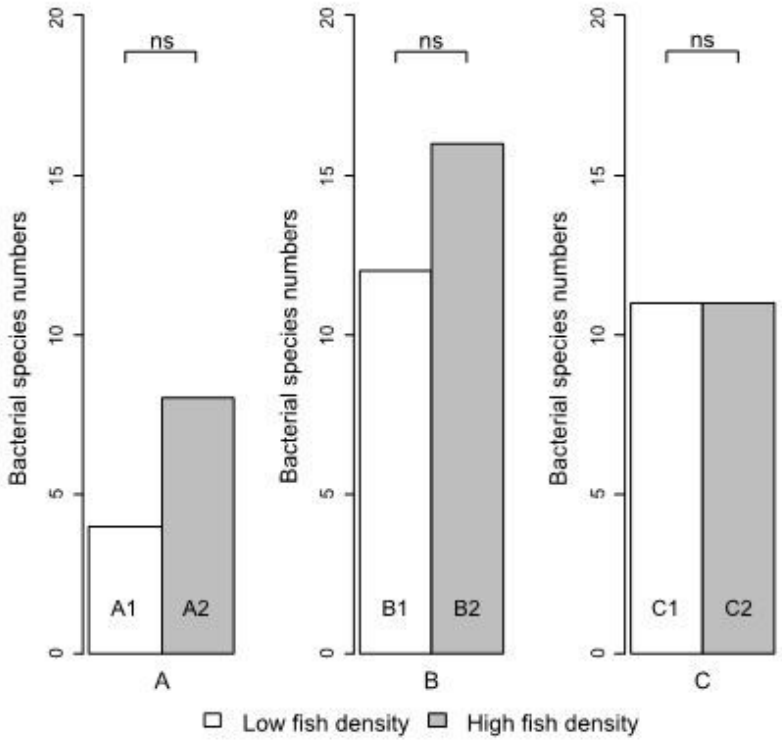

Figure 3

Total numbers of isolated bacterial species from fish stocked under low and high densities. (legend: ns = non-significant)

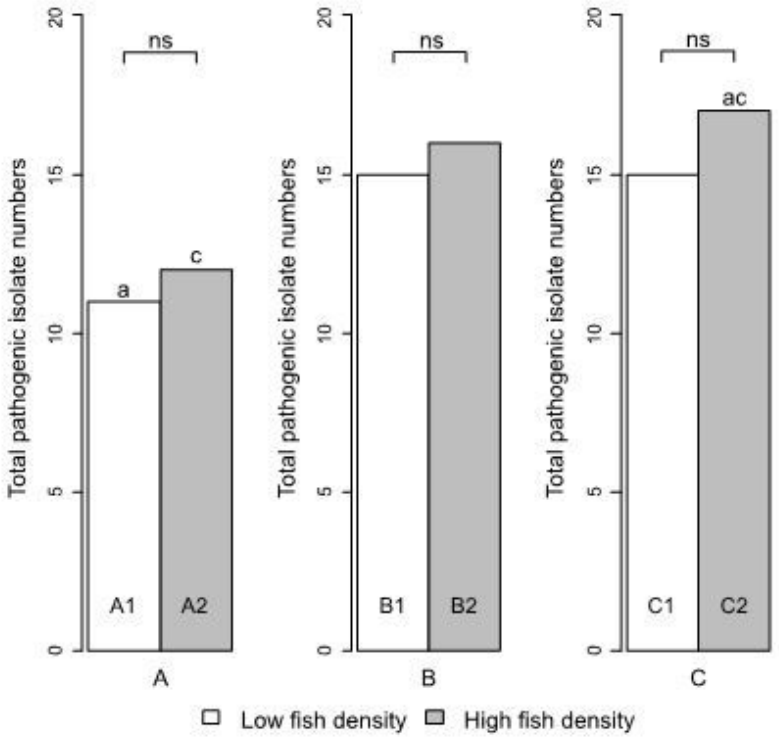

Figure 4

Total numbers of pathogenic bacteria isolated from fish stocked under low and high densities at the different sampling sites. (significance levels: a = 0.008, c = 0.03, ns = non-significant).

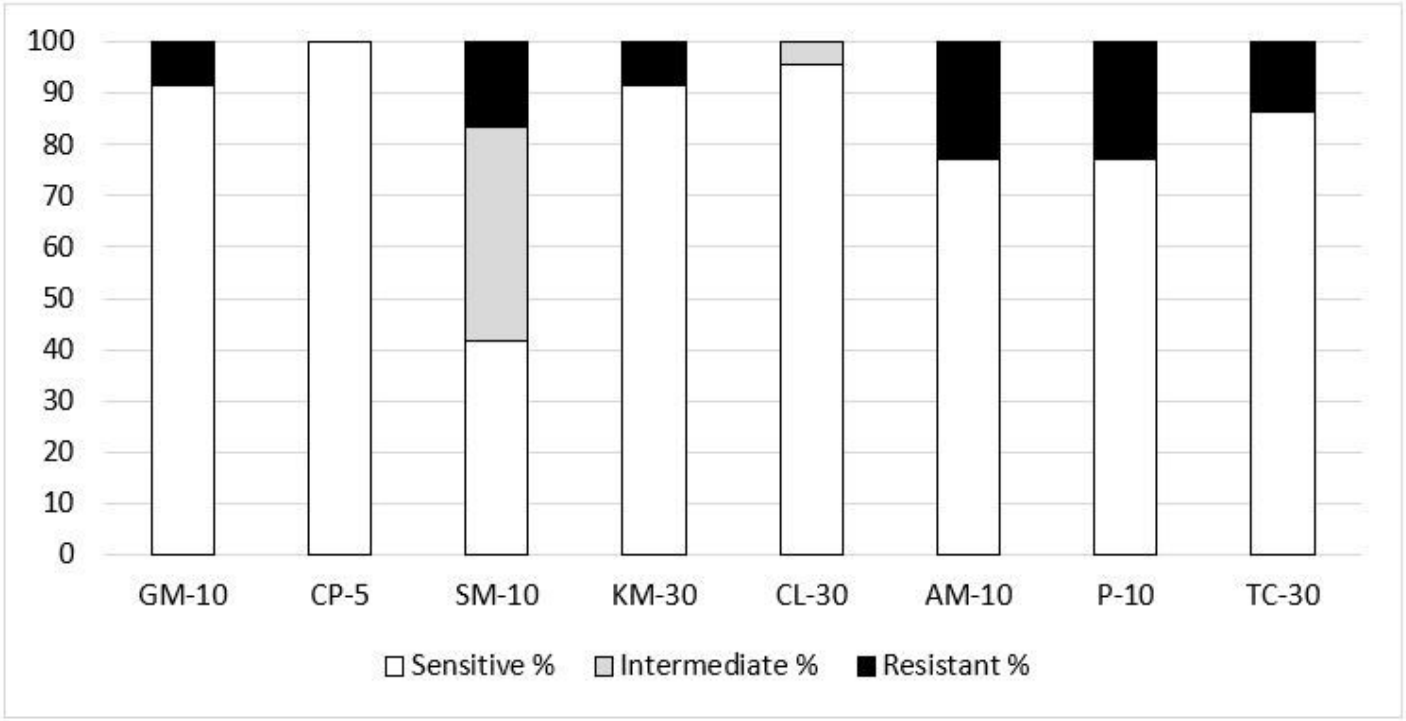

Figure 5

. Percentages of antibiotic susceptibility of the examined pathogens against the tested antimicrobial agents. (the tested sample numbers: n=12 for GM, CP, SM, KM; n=22 for CL, AM, P, TC).

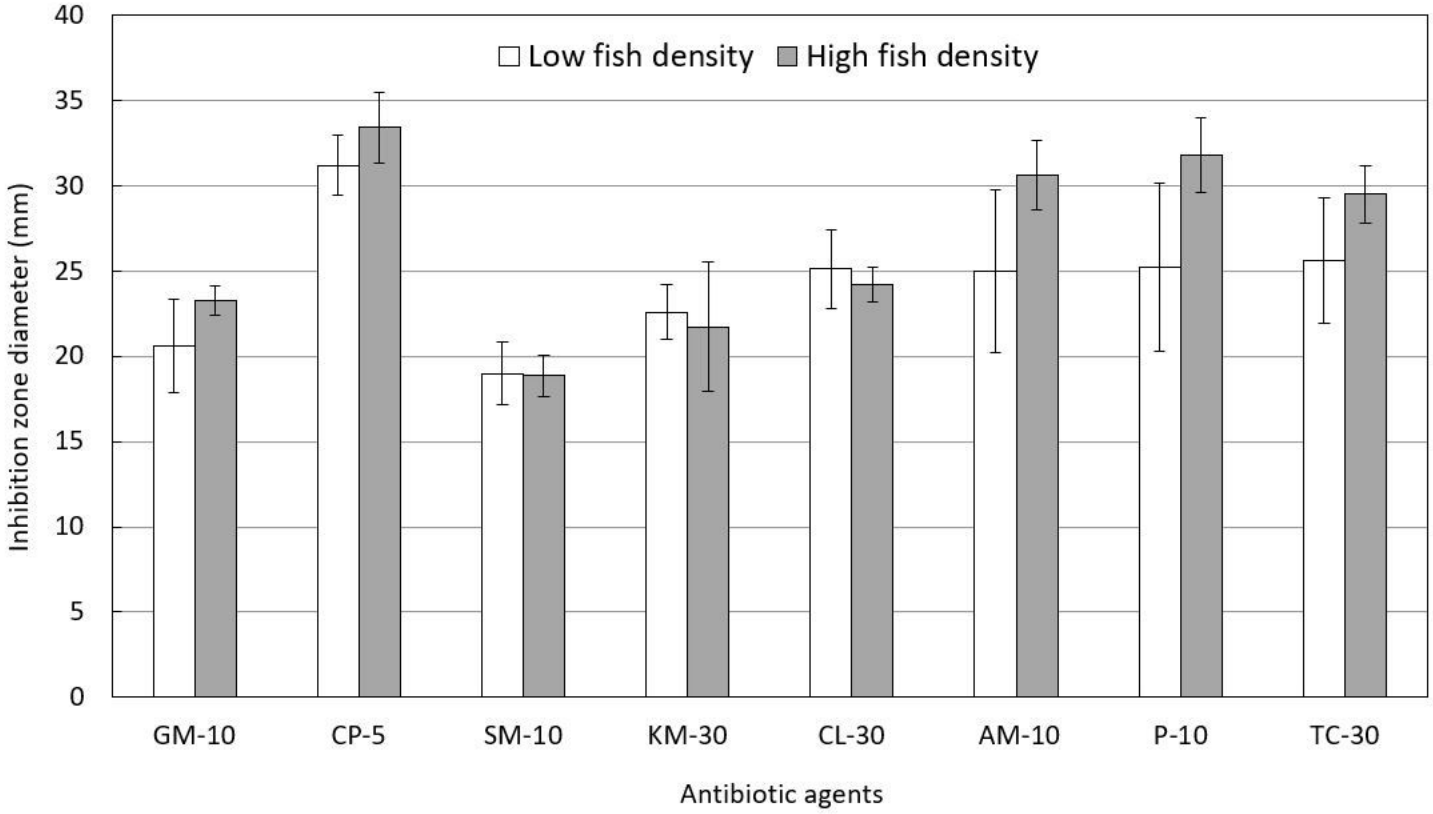

Figure 6

Bar chart of the mean value with the standard error (±SE) of susceptibility levels of pathogens isolated from fish towards eight antibiotic agents. (low fish density: n=5 for the drugs (GM, CP, SM, KM), and n=8 for the drugs (CL, AM, P, TC); high fish density: n=7 for the drugs (GM, CP, SM, KM), and n=14 for the drugs (CL, AM, P, TC)).

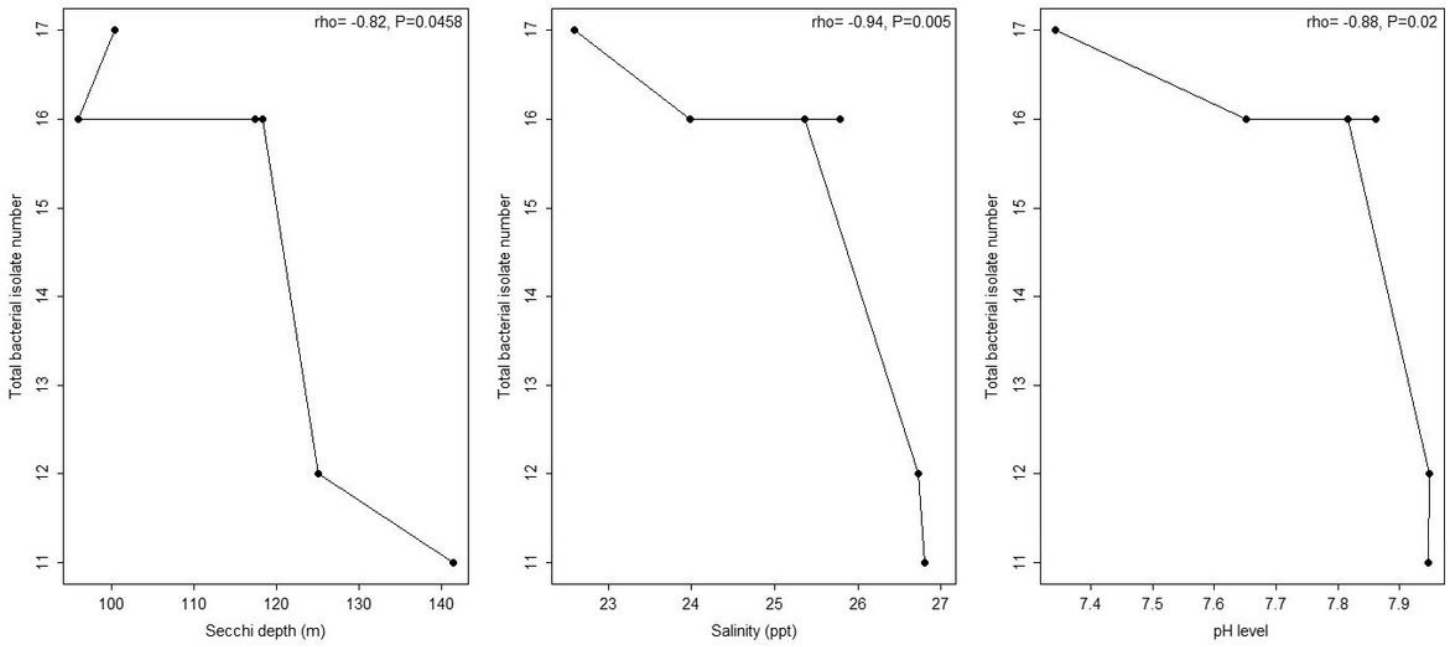

Figure 7

Monotonic correlations between selected significant water parameters and total numbers of isolates per sampling site pathogens