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Culture dependent bacteria in commercial fishes: Qualitative assessment and molecular identification using 16S rRNA gene sequencing

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Abstract

Fish contaminations have been extensively investigated in Saudi coasts, but studies pertaining to bacterial pathogens are meager. We conducted qualitative assessment and molecular identification of culture dependent bacteria in 13 fish species collected from three fishing sites and a local fish market in Jeddah, Saudi Arabia. The bacterial counts of gills, skin, gut and muscle were examined on agar plates of Macconkey's (Mac), Eosin methylene blue (EMB) and Thiosulfate Citrate Bile Salts (TCBS) culture media. Bacterial counts exhibited interspecific, locational and behavioral differences. *Mugil cephalus* exhibited higher counts on TCBS (all body-parts), Mac (gills, muscle and gut) and EMB (gills and muscle). Samples of Area I were with higher counts, concurrent to seawater and sediment samples, revealing the influence of residing environment on fish contamination. Among feeding habits, detritivorous fish harbored higher bacterial counts, while carnivorous group accounted for lesser counts. Counts were higher in skin of fish obtained from market compared to field samples, revealing market as a major source of contamination. Bacterial counts of skin were positively correlated with other body-parts indicating influence of surface bacterial biota in overall quality of fish. Hence, hygienic practices and proper storage facilities in the Jeddah fish market is recommended to prevent adverse effect of food-borne illness in consumers. *Rahnella aquatilis* (Enterobacteriaceae) and *Photobacterium damselae* (Vibrionaceae) were among the dominant species identified from fish muscle samples using Sanger sequencing of 16S rRNA. This bacterial species are established human pathogens capable of causing foodborne illness with severe antibiotic resistance. Opportunistic pathogens such as *Hafnia* sp. (Enterobacteriaceae) and *Pseudomonas stutzeri* (Pseudomonadaceae) were also identified from fish muscle. These findings indicate bacterial contamination risk in commonly consumed fish of Jeddah region.

1. Introduction

Fish are known for its exceptional health benefits particularly for defense against cardiovascular diseases (He et al., 2004; Maehre et al., 2015) and infant brain development (Boucher et al., 2011; McCann and Ames, 2005) due to the presence of long-chain polyunsaturated fatty acids (PUFAs). Despite of the nutritional benefits and culinary fondness, infection of fish by pathogenic microorganisms and other contaminants is a major concern for seafood epicureans. Fish contaminated with bacterial pathogens could cause severe foodborne illness and offset health benefits of PUFAs (Huss, 1997). Primary sources of microbial pathogens are due to anthropogenic activities that generate point and non-point pollution. There are also naturally occurring waterborne pathogens like vibrio that could cause food-borne illness in human. Monitoring of bacterial pathogens might provide early warnings to safeguard seafood consumers from threats of contamination.

Microorganisms in marine environments perform both beneficial and harmful association. They execute biogeochemical cycles in the oceans, which is a critical process in marine environments (Hewson et al., 2007). Many of the microbial species are pathogenic to human beings. They are known to produce toxins that cause lethal diseases such as paralytic shellfish poisoning, neurotoxic shellfish poisoning, diarrheic shellfish poisoning, amnesiac shellfish poisoning, and ciguatera fish poisoning (Bienfang et al., 2011; Garthwaite, 2000; Watkins et al., 2008). Microbial pollutant mainly consists of pathogenic groups of bacteria, viruses, and parasites. Bacteria represents majority of pathogens in fish that are capable of causing foodborne

diseases in human. Bacterial abundance in fish species generally varies based on environmental and biological factors. Some of the seafood commodities are inherently more risky than others owing to many factors like the nature of residing environment, species, feeding patterns, age, size, harvesting season and geographical location (Novotny et al., 2004). Hence, studies on bacterial abundance in fish are imperative at regional level due to its diverse behavior and susceptibilities.

Jeddah is the second largest city in the Kingdom of Saudi Arabia on its western seaboard. It has largest seaport in Red Sea coast, handling a total throughput of 4,700 vessels (56% container ships) and 52 million metric tons of cargo (69% discharged, 31% loaded) in 2011 (Saudi Port Authority, 2012). Rapid industrialization and urbanization in Jeddah area have raised concerns over health and environment implications. Contaminated effluents through point and non-point sources are disposed to coastal waters of Jeddah which eventually find their way into the body tissues of edible fish. Due to its exceptional nutritional benefits, fish are major dietary protein source consumed both by Saudis and expatriates in the region. In spite of significant fish consumption, adequate scientific investigations on marine pollution particularly on bacterial pathogens in Jeddah region are very scanty. Hence, it is necessary to monitor the contaminants in commonly consumed fish of Jeddah region for safeguarding public from risk of contamination. Overall objective of the study was to monitor heavy metal, organics and microbial contaminant in fish from Jeddah area. This paper deals with the bacterial contamination in commonly available and consumed fish collected from three fishing sites and local fish market in Jeddah. This study estimated bacterial abundance in various body-parts (gills, skin gut and muscle) of fishes and environmental samples (Seawater and sediment) through culture dependent methods using Macconkey's (Mac), Eosin methylene blue (EMB) and Thiosulfate Citrate Bile Salts (TCBS) agar media. Further, dominant bacterial species in the muscle of various fish species were identified through molecular techniques.

2. Materials and Methods

2.1. Sampling

Fish samples were collected from three different fishing sites (Areas 1, II and III) near Jeddah city (Fig. 1). Area I is a semi-enclosed lagoon lined with several stormwater drainage outlets, a corniche, city road network, hotels, restaurants, a private harbor, and commercial buildings within a hundred meter from the shorefront. Area II is a reef complex fronting the international port, handling mainly cargo ships. Area III is an open-water fringing reef site near the largest discharge outfall of wastewater treatment plant located south of Jeddah City. Bacterial analyses were made on 13 commonly consumed fish which were selected based on a consumption pattern survey in Jeddah region (Table 1). Fish were self-caught by baited hook and line or gillnet from various sampling areas. We also obtained fish from the central fish market in Jeddah City and a landing site at harbor (latter for *Hipposcarus harid* samples only). Collected fish samples were individually wrapped in aluminum foil and brought to the laboratory in refrigerated condition. Sediment and seawater samples were collected from all fishing sites (Areas 1, II and III) and a control site (about 70 km north from Jeddah city) for comparison. Sediment samples were collected using a van-veen grab, while seawater samples (1m below the surface) were collected using horizontal alpha water sampler. The subsamples were transferred to falcon tubes and brought to the laboratory in ice coolers.

2.2. Sample processing

Samples of gills, skin, muscle and gut of fish samples were aseptically obtained by dissection. The samples were prepared based on the methodology given by Andrews and

Hammack (2001). All the dissecting apparatuses such as scalpel, forceps, scissors, knives, mortar, pestle and glassware used in this study were sterilized with 100% ethanol and kept in hot air oven at 180 ºC for 6.0 h prior to the dissection of the each specimen. Gills were removed from both sides and homogenized to a composite sample. Skin was removed from right side of every fish behind dorsal fin after removing scales by using a sterilized knife. Muscle (flesh) samples were collected from same region using scissors and forceps. An opening was made in the body cavity with sterile scalpel and small portion of gut was removed by using a scissor. To ensure uniformity, samples were taken from same region and order. After dissection, 1 g of each dissected organs were separately homogenized by using a mortar and pestle.

The homogenized tissue, seawater and sediment samples were serially diluted before plating them for enumeration. Serially diluted samples of sediment and fish samples were plated on different selective media for the estimation of bacterial counts. Bacterial counts of seawater samples were estimated through Most Probable Number (MPN) technique.

2.3. Enumeration

Three selective isolation media, Macconkey's agar (Mac), Eosin methylene blue agar (EMB) and Thiosulfate Citrate Bile Salts agar (TCBS) were used for isolation. Isolation media (Hi media, Mumbai) were prepared by suspending Mac (51.5 g/L), EMB (36 g/L) and TCBS (89 g/L) in sterile distilled water mixed with 50% of filtered seawater. Media were completely dissolved by heating on a hot plate and sterilized by autoclaving at 15 lbs pressure and 121°C temperature for 15 minutes. TCBS media was not autoclaved but heated until boiling after suspending them in previously sterilized 50% seawater. Media were then cooled up to 50°C and aseptically poured into petri plates under sterile conditions. Enumeration of bacterial load in sediment and fish tissues were done by spread plate method. The plates were incubated in inverted position at 35±2°C for 18- 24 hours (Slaby *et al*, 1981). Bacterial colonies were counted and expressed in Colony Forming Units (CFU) per gram of given sample (Collins and Lyne, 1984). Broths of same culture media were used for estimating bacterial load in seawater samples through MPN technique.

2.4. Bacterial identification

Bacterial groups isolated from muscle samples of various fish were morphologically categorized based on their colony shape, size and color. Dominant bacterial groups in the muscle samples were sub-cultured and a single colony was subjected to colony PCR using 16S rRNA forward and reverse primers. The primers 27b F – 5' AGAGTTTGATCCTGGCTCAG 3' and 1492u R – 5' TACGGYTACCTTGTTACGACTT 3' were used for the amplification. DNA polymerase Accuprime (Invitrogen) was used to amplify 16S rRNA gene. The PCR mix was then subjected to Exosap-IT (GE Healthcare) PCR clean up protocol. The nucleotide sequence of 16S rRNA gene was determined by Sanger sequencing using Applied Biosystems 3730xl DNA Analyzer. We used the above mentioned 27b F and 1492u R primers for setting up PCR reactions using the Big Dye Terminator from ABI. Sequences from forward and reverse primers were aligned using Vector NTI software (Invitrogen) and the contigs were subjected to BLAST (Zhang et al., 2000) for nucleotide similarity search.

Phylogenetic tree was constructed using BLAST pairwise alignment tool using Fast Minimum Evolution method (Desper and Gascuel, 2004). The genus and species were identified based on lowest E-value in BLAST. Multiple sequence alignments of nucleotides were performed using Clustal Omega web tool (Sievers et al., 2011). Sequences of different genus and those with differences within same genus were submitted to GenBank/NCBI database for accession number (Table 2).

Phylogentic analyses of the isolates were carried out using Phylogeny (Dereeper et al., 2010; http://www.phylogeny.fr) using tools such as MUSCLE (Edgar, 2004) for multiple sequence alignment, Gblocks for curating sequences (Castresana, 2000), PhyML for constructing tree (Guindon, 2003; Anisimova, 2006) and TreeDyn for analyses of tree (Chevenet, 2006).

2.5. Statistical analysis

Statistical analyses and graphical presentations were performed using the SPSS Statistics 20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) and Microsoft Excel spreadsheet software packages.

3. Results

3.1. Environmental samples

Mean bacterial counts of seawater and sediment samples are depicted in the Fig. 2. Counts in seawater were higher in Area I (8.45, 1.91 and 3.8 $\times 10^5$ MPN mL^{-1} respectively in Mac, EMB and TCBS), while lowest loads were detected in control site (0.072, 0.066 and 0.094 $\times 10^5$ MPN mL^{-1} respectively). The pre-condition test Levene's statistics was non-significant (P>0.05), indicating homogeneity of variances between sampling sites. Comparisons by ANOVA exhibited significant variation (P<0.01) in bacterial counts on Mac (F $_{(3, 8)}$ = 40.01), EMB (F $_{(3, 8)}$ = 32.01) and TCBS (F $_{(3, 8)}$ = 64.35). Higher bacterial counts of sediment were noted in Area I (600, 412 and 526 $\times 10^5$ CFU g^{-1} respectively on Mac, EMB and TCBS), while lowest in control site (0.38, 0.30 and 0.22 $\times 10^5$ CFU g^{-1} respectively). Comparisons by ANOVA exhibited significant variation in counts on Mac (F $_{(3, 8)}$ =129, P<0.01) and TCBS (F $_{(3, 8)}$ =345, P<0.01) between sites. Levene's statistics was significant (P<0.05) for counts on EMB. Hence, instead of F statistic robust tests Welch and Brown-Forsythe statistics was conducted. This showed significantly different counts between stations (Welch F $_{(3, 4.28)}$ = 64.54 and Brown-Forsythe F $_{(3, 2.14)}$ = 242; P<0.01). Turkey HSD post hoc tests revealed that bacterial load of seawater and sediment in control site varied significantly from other stations (P<0.05). This was well supported by Scheffe and Tamhane statistics.

3.2. Fish samples

3.2.1. Counts on Mac Agar

Mean bacterial counts of various body-parts on Mac agar are shown in Fig. 3. Counts of gills were higher in *Mugil cephalus* from Area I (491$\times 10^5$ CFU g^{-1}) and lower in market samples of *Carangoides bajad* (0.15$\times 10^5$ CFU g^{-1}). Counts of skin were higher in market samples of *Cephalopholis argus* (615$\times 10^3$ CFU g^{-1}) and lower in *Variola louti* from Area II (2.7$\times 10^3$ CFU g^{-1}). Counts of muscle samples were higher in *M. cephalus* from Area I (238$\times 10^3$ CFU g^{-1}), whereas undetected in *Lethrinus lentjan* and *V. louti* from Area II, market and field samples of *Chanos chanos* and market samples of *Lutjanus bohar* and *Aphareus rutilans*. Bacterial counts of gut were higher in market samples of *M. cephalus* (220$\times 10^5$ CFU g^{-1}), whereas lower in *V. louti* from Area II (0.55$\times 10^5$ CFU g^{-1}).Comparison of bacterial counts on Mac agar between species revealed significant variations for gut (Welch F $_{(12, 19.5)}$ = 5.56, P<0.01; Brown-Forsythe F $_{(12, 10.7)}$ = 3.28, P <0.05) and gills (Welch F $_{(12, 20.1)}$ = 11.51, P<0.01; Brown-Forsythe F $_{(12, 33)}$ = 7.24, P <0.01) but non- significant for skin (Welch F $_{(12, 19.4)}$ = 1.71, P>0.05; Brown-Forsythe F $_{(12, 18.2)}$ = 1.81, P >0.05). Counts of muscles, compared by ANOVA, exhibited significant variation between species (F $_{(9, 41)}$ =4.75, P<0.01).

3.2.2. Counts on EMB Agar

Mean bacterial counts on EMB agar for different body-parts are shown in Fig. 4. Counts of gills were higher in *M. cephalus* from Area I (146$\times 10^5$ CFU/g) and lower in *Lethrinus lentjan* from Area II (0.05$\times 10^5$ CFU/g). Counts of skin were higher in *V. louti* from market (308$\times 10^3$

CFU/g), whereas undetected for same species from Area II. In muscles, bacterial counts were higher in market samples of *M. cephalus* (41.2×10^3 CFU/g), while undetected in *L. lentjan* and *V. louti* (both from Area II), *C. chanos* (Area I and market), *L. bohar* and *Aphareus rutilans* (both from market). Counts of gut samples were higher in *L. bohar* from market (32.3×10^5 CFU/g) and lowest in *V. louti* from Area II (0.09×10^5 CFU/g). Bacterial counts in body-parts isolated on EMB agar, compared using robust tests revealed significant variations for muscle (Welch $F_{(9, 12.6)}$ = 8.03, P<0.01; Brown-Forsythe $F_{(9, 13.2)}$ = 3.57, P <0.05) gut (Welch $F_{(12, 19.4)}$ = 7.61; Brown-Forsythe $F_{(12, 46.7)}$ = 2.67; P <0.01), gills (Welch $F_{(12, 18.6)}$ = 5.41; Brown-Forsythe $F_{(12, 37.3)}$ = 4.51; P<0.01) and skin (only with Welch $F_{(12, 19.5)}$ = 3.31, P<0.01).

3.2.3. Counts on TCBS Agar

Bacterial counts of body-parts on TCBS agar for different fish species are given in Fig. 5. Counts of gills were higher in market samples of *M. cephalus* (351×10^5 CFU g^{-1}) and lower in *C. bajad* (0.04×10^5 CFU g^{-1}) from same source. Counts of skin were higher in *M. cephalus* from Area I (696×10^3 CFU g^{-1}) and lower in *C. argus* from Area II (1.1×10^3 CFU g^{-1}). Mean counts of muscle were higher in *M. cephalus* from Area I (61.5×10^3 CFU g^{-1}), whereas it was undetected in *V. louti* from Area II, market and field samples of *C. chanos* and market samples of *C. bajad*, *L. bohar* and *A. rutilans*. Counts of gut samples were higher in *M. cephalus* from Area I (206×10^5 CFU g^{-1}) and were lower in *V. louti* from Area III (0.52×10^5 CFU g^{-1}). Comparison of bacterial counts on TCBS agar between various species revealed significant variations in skin (Welch $F_{(12, 19.6)}$ = 4.33, P<0.01; Brown-Forsythe $F_{(12, 17.5)}$ = 2.60, P<0.05), gut (Welch $F_{(12, 20.0)}$ = 200; Brown-Forsythe $F_{(12, 21.4)}$ =2.44; P<0.05), gills (Welch $F_{(12, 18.7)}$ =10.4, P<0.01; Brown-Forsythe $F_{(12, 38.05)}$ = 5.57; P<0.05) and muscle (Welch $F_{(9, 13.7)}$ = 10.8, P<0.01; Brown-Forsythe $F_{(9, 23.8)}$ = 6.10, P<0.01).

3.3. Locational differences

Bacterial counts in fish species grouped based on their sampling sites are given in Fig. 6. Counts of gut, gills and muscle on Mac and TCBS were higher in fish from Area I, while those for skin were in market samples. Counts of gut, gills and skin on EMB were higher in Area III, while that for muscle were in Area I samples. Comparison of bacterial counts on Mac agar between sampling sites exhibited significant variation (P<0.01) for skin ($F_{(3, 71)}$ = 7.52), gut ($F_{(3, 71)}$ =5. 18), and gills ($F_{(3, 71)}$ = 5.57). Counts on EMB agar showed significant variation (P<0.01) for skin (Welch $F_{(3, 17.3)}$ = 7.50; Brown-Forsythe $F_{(3, 30.7)}$ = 6.13), gills (Welch $F_{(3, 23.2)}$ = 8.43; Brown-Forsythe $F_{(3, 49.5)}$ = 11.4) and gut (ANOVA $F_{(3, 71)}$ = 4.67, P<0.01). Counts on TCBS agar displayed significant variation (ANOVA; P<0.01) in skin ($F_{(3, 71)}$ =5.64), gut ($F_{(3, 71)}$ = 4.48) and gills ($F_{(3, 71)}$ = 8.28). Counts of muscle sample on the all the three media exhibited non-significant variation (P>0.05) between the sampling sites.

3.4. Behavioral differences

Bacterial counts in various body-parts of fish grouped based on their feeding behavior are presented in Fig. 7. Counts of gills and muscle showed higher values in detritivorous fish on all tested media. Same fish group showed higher counts for skin and gut samples on Mac and TCBS. Higher counts on EMB agar for the said samples were observed respectively with carnivorous and herbivorous fish groups. Comparison of bacterial counts on TCBS agar between different fish groups were significant for skin (ANOVA $F_{(2, 72)}$ = 6.23; P<0.01), gut (ANOVA $F_{(2, 72)}$ = 3.98; P<0.05), gills (Welch $F_{(2, 19.1)}$ = 57.9; Brown-Forsythe $F_{(2, 44.9)}$ = 40.09; P<0.01) and muscle (Welch $F_{(2, 30.6)}$ = 44.5; Brown-Forsythe $F_{(2, 25.5)}$ = 8.63; P<0.01). Comparison of counts on Mac agar for different fish groups showed significant variation for gut (ANOVA $F_{(2, 72)}$ = 6.48; P<0.01), gills (Welch $F_{(2, 19.9)}$ = 45.5; Brown-Forsythe $F_{(2, 42.9)}$ = 26.71, P<0.01) and

muscle (Welch F $_{(2, 24.85)}$ = 25.11; Brown-Forsythe F $_{(2, 27.53)}$ = 7.05, P<0.01), while it was non-significant for skin (P>0.05). Comparison of counts on EMB agar exhibited significant variation for muscle (Welch F $_{(2, 28.7)}$ = 33.3; Brown-Forsythe F $_{(2, 25.25)}$ = 7.16, P<0.01) and gills (only Welch test (F $_{(2, 11.6)}$ = 6.16, P<0.05). Counts of skin and gut on EMB displayed non-significant variation (ANOVA; P>0.05) between the fish groups.

3.5. Identification

16S rRNA gene fragment amplified using 27b F and 1492u R universal primers were around 1500bp which covers 97% of the total. Obtained sequences were aligned using Vector NTI and inspected by eye. Further, it was manually edited to remove ambiguities based on PHRED scores and chromatogram. Fully aligned contig sequences were analyzed online with BLASTN for nucleotide similarity against 16S rRNA database. Bacterial species with hits of lowest E-value were identified and unique organisms were selected using clustal omega tool. These were submitted in GenBank/NCBI database (Table 2). Bacterial species of g-proteobacteria group, constituting the families Vibrionaceae, Enterobacteriaceae, Aeromonadaceae, Moraxellaceae, Pseudomonadaceae and Pseudoalteromonadaceae were identified. Phylogenetic analysis (Fig. 8) revealed *Rahnella aquatilis* (strain NA06) and *Hafnia* sp. (strain NA08) are closely related bacterial species. The strains of *Aeromonas* sp. showed little differences in its branch length. However, the bootstrap values showed that identified species were not very closely related.

4. Discussion

Investigations on bacterial loads of marine fish are imperative for contamination assessment studies. Bacterial communities are usually very complex in their association and challenging for isolation. Consequently, a meager percentage of them could be cultured under laboratory conditions and only few of them are taxonomically identified. There is no single method to enumerate and estimate all bacterial population. However, culture dependent heterotrophic bacteria population is conventionally estimated on solid media plates as pollution indicator in marine environment (Gonzalez and Moran, 1997; Pinhassi et al., 1997). Regardless of paucities and methodological limitations in laboratory culture, information on bacterial species that form colonies on media plates provide valuable insights on prevalence of pathogenic microorganisms in any test sample. Food contamination studies generally examine indicator bacterial groups, which is practically easier and cost effective. Several of indicator bacteria are not pathogenic themselves; however their abundance postulates contamination risks.

This study quantified bacterial load in seawater, sediment and 13 edible fish samples collected from three distinct fishing grounds and central fish market of Jeddah (table 1). Bacterial counts of seawater and sediment varied significantly between sampling sites. Area I exhibited higher bacterial counts, while lower counts were recorded from control site. Agreeing with Scheffe and Tamhane statistics, Turkey HSD post hoc tests revealed that bacterial counts of fishing sites varied significantly (P<0.05) from control site (Fig. 4). Fishing sites were located in close vicinity to the sewage disposal regions of Jeddah city, hence higher bacterial counts in fishing sites could be attributed to this. Recent report cited severe bacterial contamination in many coastal regions of Jeddah due to untreated discharges to the sea (JEA, 2013). Control site (70 km away from fishing site) recorded lesser bacterial load, since unpolluted region harbor less bacterial load. Bacterial load could be influenced by abiotic factors of local environment (Madigan et al., 2000), but such correlation is not attempted in this study. Lack of previous records impedes temporal evaluation of bacterial contamination in the fishing sites. Previous study at Al- Nawrus lagoon, a closer location to fishing sites of our study, recorded heterotrophic bacterial counts between 10^5 - 10^6 CFU mL^{-1} and total coliform counts of 10^2 CFU/100 mL

(Turki and Mudarris, 2008), which is relatively lesser than present study. Results of our study ascertain existence of bacterial contamination in seawater and sediment of coastal Jeddah. Bacterial load in tested sites were higher in sediment than seawater samples, withstanding the fact that a comparison is improper, as the counts are expressed in CFU and MPN respectively. Higher bacterial counts of sediment might be due to its rich organic content and less stressors like sunlight and predation (Craig et al., 2002; Rodrigues et al., 2011; Rose et al., 2001; Rubentschik et al., 1936). However, entry of pathogens is anticipated to be through seawater which receives several disease-causing pathogens from animal and human wastes from wadi runoffs, infiltration and subsurface flow, discharges and flood water from inlands of Jeddah region. Microbes received by seawater sink in sediments and act as reservoir for pathogens which might get accumulated in residing fishes.

Our results displayed interspecific variation in bacterial counts in fish. The tested agar media Mac, EMB and TCBS supports growth of total coliform, *E. coli* and vibrio respectively. Coliform and *E. coli* are associated with many chronic illnesses and have been used as standard indicators of pollution in near-shore marine environments. Vibrios are dominant bacterial species in sewage discharges and have been linked with foodborne diseases including pandemic diarrhea outbreaks (Austin, 2010). Total coliform, *E. coli* and vibrio in fish have been considered as indicator of bacterial contamination (Adebayo-Tayo et al., 2011; Akinyemi and Buoro, 2011; E1-Zanfaljz and Ibrahim, 1982; Jha et al., 2010). We noted higher bacterial counts in *M. cephalus* on TCBS (all body-parts), Mac (gills, muscle & gut) and EMB (gills & muscle). Similar to this observation, a study from Lagos lagoon (Nigeria) detected higher bacterial counts in skin, buccal cavity and gills of *M. cephalus* than other tested fishes (Akinyemi and Ajisafe, 2011). Fish intake bacterial pathogen from seawater, sediment and food (Sugita et al., 1988) and hence their abundance is largely influenced by residing environment (Shewan and Hopps, 1967). Indeed, fishes are actively swimming marine organisms. Nevertheless, we noted higher bacterial counts in fish from Area I in concurrent with sediment and seawater that exhibited rich bacterial abundance than other sampling sites (Fig. 6). There was significant variation (P<0.01) in bacterial counts of skin samples on Mac and TCBS agar between sampling sites indicating influence of residing environment. Bacterial counts of skin were positively correlated with other body parts. Pearson correlation showed significant correlation of skin with gills (r = 370, P<0.01; r = 456, P<0.01; r = 256, P<0.05), muscle (r = 374, P<0.01; r = 346, P<0.01; r = 287, P<0.05), and gut (r = 291, P<0.05; r = 545, P<0.01; r = 298, P<0.01) respectively on Mac, EMB and TCBS agar. This reveals influence of surface bacterial biota in overall quality of fish. Counts on TCBS and Mac agar of skin were particularly higher in market fish than field samples. For instance skin samples of species such as *C. argus*, *E. tauvia*, *M. cephalus* and *V. louti* exhibited significantly increased counts on TCBS and Mac agar, than its counterparts collected from field. This might be due to unsanitariness of market premises and unhygienic fish handling practices in Jeddah fish market. Food-borne diseases are associated with deficient hygiene practices and cross-contamination from equipment and food handlers during processing and storage. Besides, bacterial counts of aquatic animals multiply after their death (Faghri et al., 1984). Market samples were obtained after few or sometimes several hours of catch. Hence, natural decay along with unhygienic conditions of premises resulted in higher bacterial counts of market fish samples.

In general, detritivorous fish were with higher bacterial counts, while carnivorous fish accounted for lesser load. Higher total coliforms counts in detritivorous fish were noted in fresh water species (Rahman et al., 2010). Intestinal microbes are major causative agent for seafood

spoilage (Kaneko, 1971). Our data for counts on Mac and TCBS agar of gut samples exhibited significant variation between the fish groups (P<0.05). This reveals that intestinal bacterial load in fish are influenced by feeding habit. However, this assumption requires further investigation.

Dominant species identified from muscle samples include established pathogenic bacteria, opportunistic pathogens and also non-pathogenic form. The bacterial species of the family Vibrionaceae, Enterobacteriaceae, Aeromonadaceae, Moraxellaceae, Pseudomonadaceae and Pseudoalteromonadaceae were identified. Vibrionaceae family was represented by *Photobacterium damselae* and *Vibrio harveyi*. Species *Photobacterium damselae* (formerly *Vibrio damsela*) is a dominant halophilic bacterium abundant in marine environments capable of causing infections and fatal disease in marine animals and humans. It is an established human pathogen with most of reported infections are on wounds inflicted during the handling of fish, exposure to seawater and marine animals, and ingestion of raw seafood (Asato and Kanaya, 2004; Morris et al., 1982;). In some cases the infection leads to a fatal outcome (Yamane et al., 2004). *V. harveyi* is a free-swimming bacterium in tropical marine waters and dominant gut microflora (Austin and Zhang, 2006). This species is categorized as primary or opportunistic pathogen but not an established human pathogen.

Enterobacteriaceae family was represented by *Rahnella aquatilis* and *Hafnia* sp. *R. aquatilis,* recognized in 1976–1979 (Gavini et al., 1976; Izard et al., 1979), was isolated from open water reservoirs, soil, clinical samples and food materials (Gras et al., 1994; Farmer, 1984; Funke and Rosner, 1995; Lindberg et al., 1998). They are opportunistic pathogens causing a wide spectrum of life-threatening infections in humans mainly affecting gastrointestinal tract, urinary tract, respiratory organs and cardiovascular system (Zdorovenko et al., 2004). Besides being highly pathogenic, *R. aquatilis* are naturally resistant to many commercial antibiotics (Stock et al., 2000). *Hafnia alvei* is the only species identified under genus *Hafnia*. Although, our sequence data doesn't confirm the species, the genus *Hafnia* is not normally pathogenic to humans. It has received attention from medical community due to its commensalism with human gastrointestinal tract and its resistance to antibiotics. *H. alvei* was originally isolated and identified at the International Centre for Diarrhoeal Disease Research, Bangladesh (Albert et al., 1991). However, 16S ribosomal DNA sequencing and DNA-DNA pairing studies have validated the strains a *Escherichia*. Hence, a new species, *E. albertii*, has been proposed instead of *H. alvei* (Huys et al., 2003). Thus, the role of this species as a gastroenteritis and diarrheal disease causing agent is yet to be confirmed.

Moraxellaceae family was represented by two morphologically different strains (NA03 and NA05) of *Psychrobacter faecalis*, which is not associated with foodborne illness. It is a relatively new species, first identified from bioaerosol generated by cleaning of a pigeon faeces contaminated room isolated using MacConkey agar at a 36°C (Kämpfer et al., 2002). This study also used same inoculation media and incubated at a similar temperature range. The genus *Psychrobacter* has been isolated from gills and skin of fish, poultry, food and clinical sources (Juni and Heym, 1986).

Identified species of Aeromonadaceae family includes *Aeromonas* sp. (species not identified) and *A. salmonicida*. Genus *Aeromonas* are ubiquitous in aquatic environments and have been isolated from patients with gastroenteritis (Altwegg and Geiss, 1989; Joseph, 1996), indicating virulence of this species in causing foodborne illness due to its capability of producing extracellular toxins and enzymes (Gosling, 1996; Howard et al., 1996). Enterotoxin-producing *Aeromonas* spp. have shown significant correlations with diarrhea patients (Bloom and Bottone, 1990; Joseph, 1996). Although several species of *Aeromonas* are known pathogens, the species

A. salmonicida has not been associated with human infection (USEPA, 2006), but it is a known fish pathogen.

Pseudomonadaceae family was represented by *Pseudomonas stutzeri*, which is rarely found in humans and known to cause illness. Human illness associated studies on *P. stutzeri* found that many cases were probable hospital acquired infections during surgical procedures and/or due to immune compromised condition (Reisler and Blumberg, 1999). Hence, it is clear that *P. stutzeri* is an opportunistic pathogen of low virulence and cannot been associated with foodborne illness. Pseudoalteromonadaceae is represented by *Pseudoalteromonas* sp. which is formerly classified in the genus Alteromonas. This species is ubiquitous in marine environment associated with eukaryotic hosts (Gauthier et al., 1995), capable of producing the lethal tetrodotoxin (Simidu et al., 1990). However, none of its species are established human pathogen.

5. Summary

We studied general pattern of bacterial contamination in the commercial marine fishes from Jeddah region. The results exhibited interspecific, locational and behavioral difference in the bacterial load of the fish. The accepted limit of aerobic bacterial counts for fresh fish is proposed between 5×10^5 - 5×10^7 CFU g^{-1} and that of *E. coli* between 1×10^1 - 5×10^2 CFU g^{-1}(ICMFS, 1986). According to Saudi Arabia Standards Organization (SASO) for bacterial load of foodstuffs (No 1556; SASO, 1998), the level in meat products should not exceed 10^6 CFU g^{-1}, and *E. coli* counts should be less than 10^2 CFU g^{-1}. This study recorded higher bacterial counts than the accepted limit in fish samples. 16S rRNA sequences identified pathogenic bacteria from Vibrionaceae and Enterobacteriaceae in the tested fish samples. Dominant species such as *Rahnella aquatilis* and *Photobacterium damselae* are known human pathogens capable of causing foodborne illness with severe antibiotic resistance Opportunistic pathogens such as *Hafnia* sp. (Enterobacteriaceae) and *Pseudomonas stutzeri* (Pseudomonadaceae) were also identified. Dominant existence of these bacterial species in fish muscle samples are of major concern. Some of these species are autochthonous in marine environment with ubiquitous distribution due to its halophilic nature.The overall result of this study indicates the risk of bacterial contamination among seafood consumers in Jeddah region. Hence, periodic monitoring of bacterial population along with detailed studies on community structure is recommended through this study.

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Table 1 Fish species used for microbiological analysis, their local name(s), feeding habit and
sampling site.

No.	Species	Local Name	Feeding behavior	Areas			Market	Landing site
				I	II	II		
1	*Chanos chanos*	Salmani	Herbivor	√			√	
2	*Mugil cephalus*	Arabi, Bori	Detritivor	√			√	
3	*Carangoides bajad*	Bayad	Carnivor		√		√	
4	*Lethrinus lentjan*	Shaoor, Sheiry	Carnivor		√	√	√	
5	*Epinephelus tauvina*	Tauvina	Carnivor		√		√	
6	*Plectropomus pessuliferus*	Najil	Carnivor		√		√	
7	*Variola louti*	Louti, Sharef	Carnivor		√	√	√	
8	*Cephalopholis argus*	Hamour	Carnivor		√	√	√	
9	*Siganus rivulatus*	Sijan, Safi	Herbivor				√	
10	*Lutjanus bohar*	Bohar	Carnivor				√	
11	*Plectropomus truncatus*	Tarathi	Carnivor				√	
12	*Aphareus rutilans*	Mosa, Faris	Carnivor				√	
13	*Hipposcarus harid*	Harid	Herbivor				√	√

Table 2: Bacterial species identified based on 16S rRNA gene and its acquired GeneBank accession numbers

No.	Organism	GenBank Accession number
1	*Pseudomonas stutzeri* strain NA01	KJ563264
2	*Vibrio harveyi* strain NA02	KJ563265
3	*Psychrobacter faecalis* strain NA03	KJ563266
4	*Aeromonas salmonicida* strain NA04	KJ563267
5	*Psychrobacter faecalis* strain NA05	KJ563268
6	*Rahnella aquatilis* strain NA06	KJ563269
7	*Photobacterium damselae* strain	KJ563270
8	*Hafnia* sp. strain NA08	KJ563271
9	*Pseudoalteromonas* sp. strain NA09	KJ563272
10	*Aeromonas* sp. strain NA10	KJ563273

Figure Captions

Figure 1 Map showing study area with different sampling sites (Area I-III, Landing site and fishmarket). Control site (not in the figure) is about 70 km away north from Jeddah city

Figure 2 Boxplots for bacterial counts in sediment and seawater from various sampling sites isolated on three different culture media

Figure 3 Bacterial counts on MacConkey (Mac) agar by samples of gills ($\times 10^5$ CFU g^{-1}), skin ($\times 10^3$ CFU g^{-1}), gut ($\times 10^5$ CFU g^{-1}), and muscles ($\times 10^3$ CFU g^{-1}) of fish species from various sources. (Mean values of three individuals; species is coded by genus initial and first three letters of specific name; source is coded by A1□A3, M, and L corresponding to Areas I□III, market, and landing site respectively)

Figure 4: Bacterial counts on Eosin Methylene Blue (EMB) agar by gills ($\times 10^5$ CFU g^{-1}), skin ($\times 10^3$ CFU g^{-1}), gut ($\times 10^5$ CFU g^{-1}), and muscles ($\times 10^3$ CFU g^{-1}) of different fishes collected from various sampling sites. (Mean values of three individuals; sample labels are same as the figure 3)

Figure 5: Bacterial counts on Thiosulfate-Citrate-Bile Salts (TCBS) agar by gills ($\times 10^5$ CFU g^{-1}), skin ($\times 10^3$ CFU g^{-1}), gut ($\times 10^5$ CFU g^{-1}), and muscles ($\times 10^3$ CFU g^{-1}) of different fishes collected from various sampling sites. (Mean values of three individuals; sample labels are same as the figure 3)

Figure 6 Bacterial counts on Mac, EMB and TCBS agar exhibited by various body parts of fish species grouped based on sampling sites (Landing site in the graphs are mentioned as harbor)

Figure 7 Bacterial counts on Mac, EMB and TCBS agar exhibited by various body parts of fish species grouped based on feeding behavior

Figure 8: Phylogenetic tree constructed for the identified bacterial species isolated from muscle of the fish species from Jeddah region. Bar represents 2 % sequence divergence.
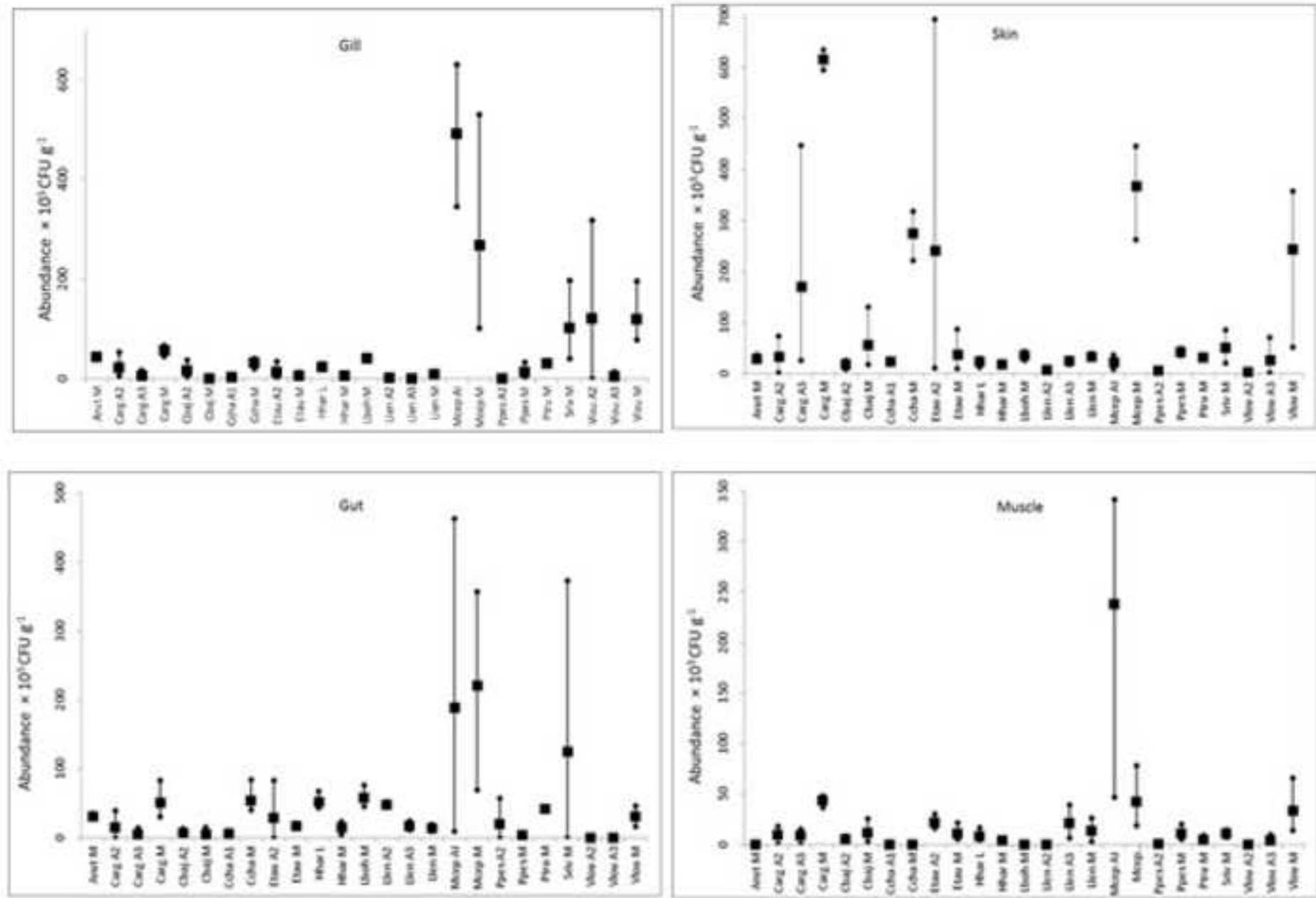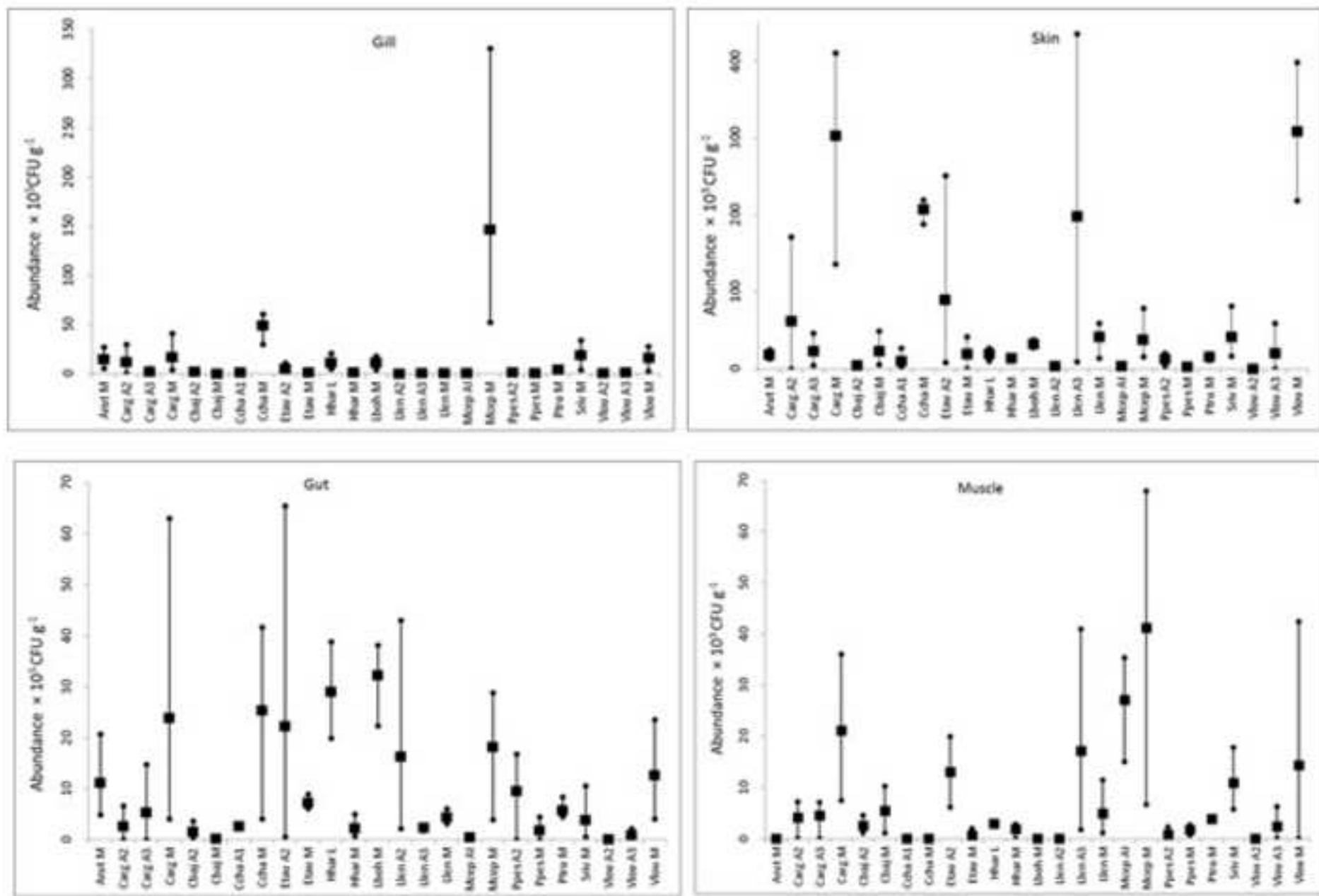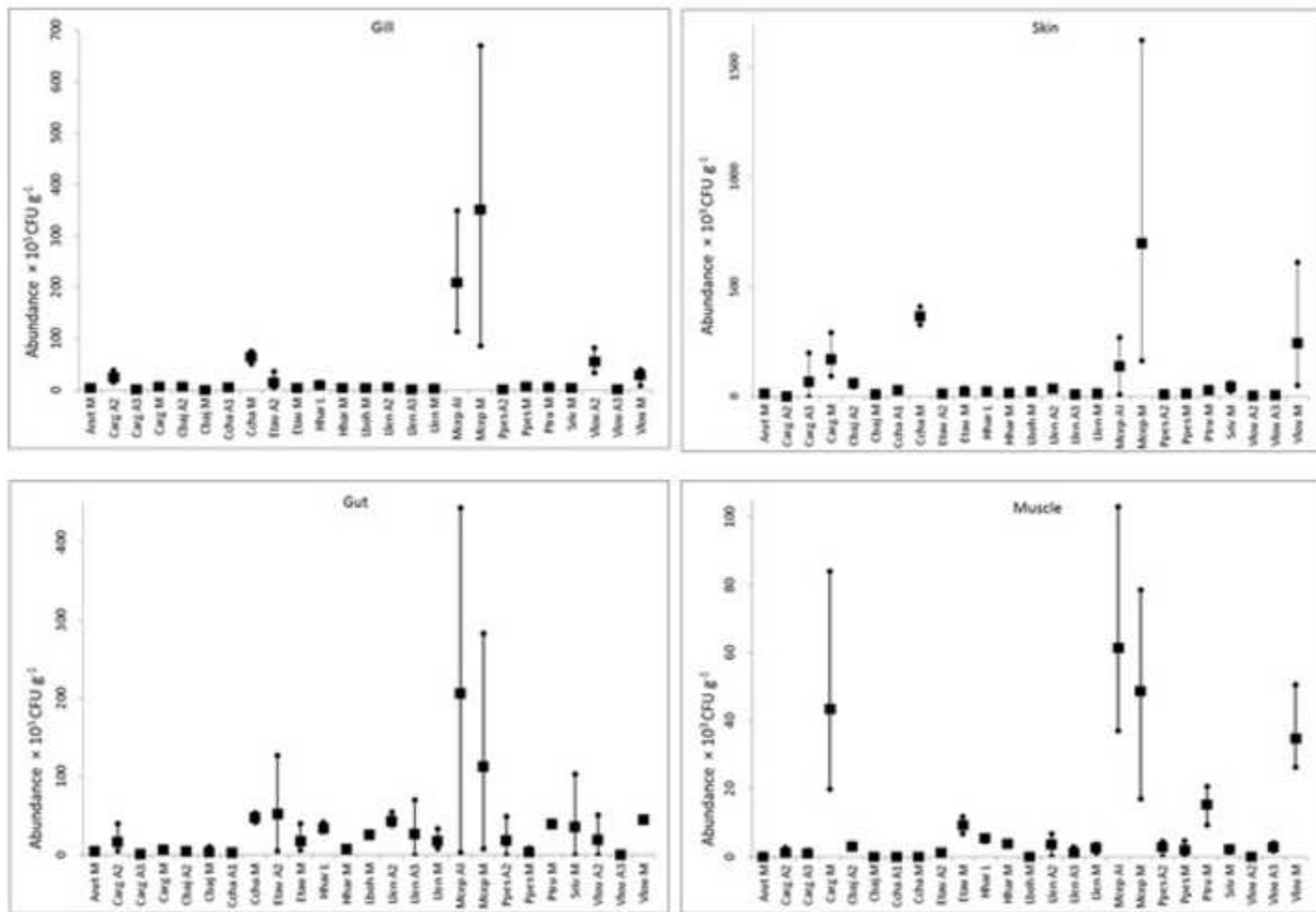
Figure 1

Figure 2

Seawater

Sediment

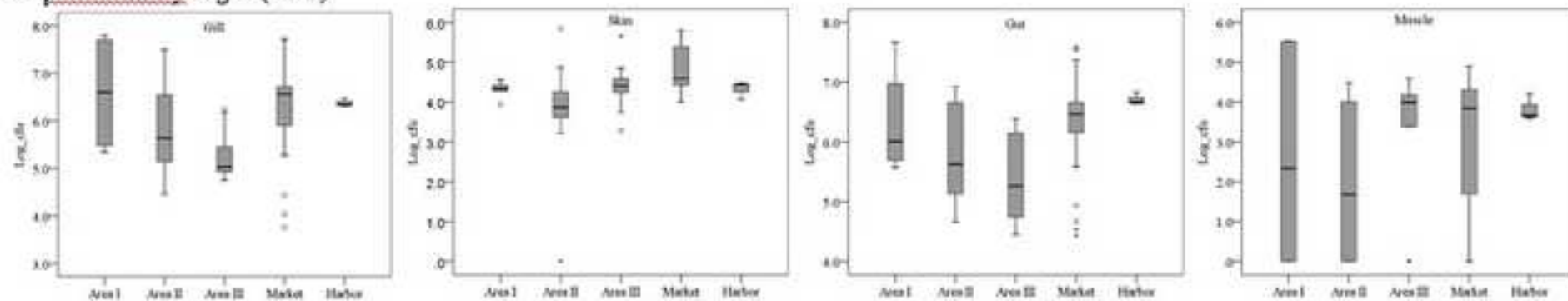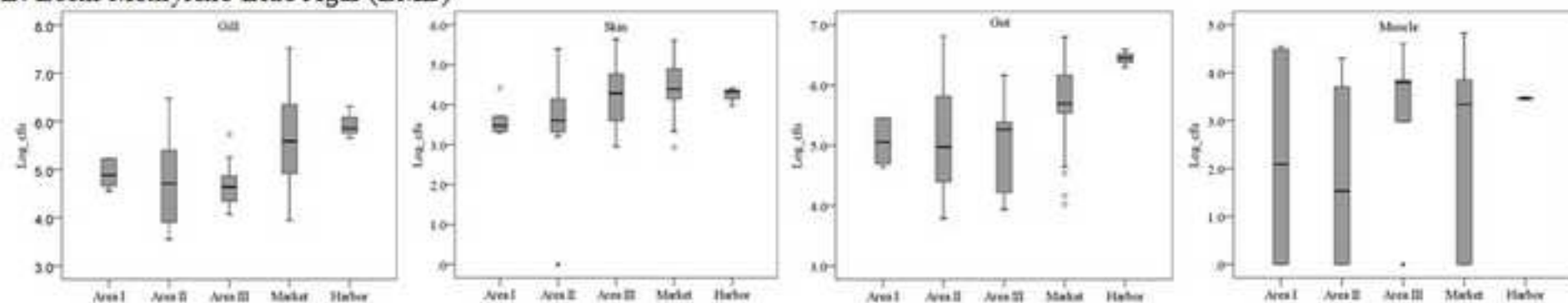**Figure 3**

Figure 4

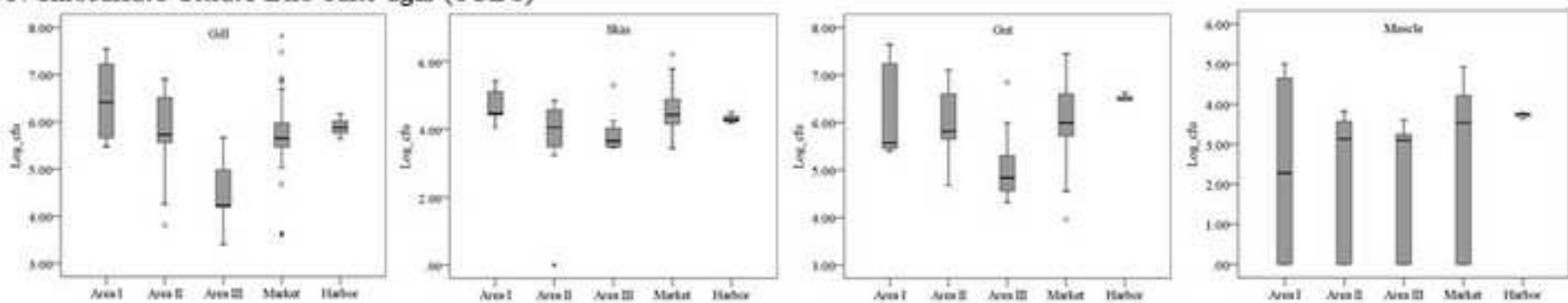**Figure 5**

Figure 6
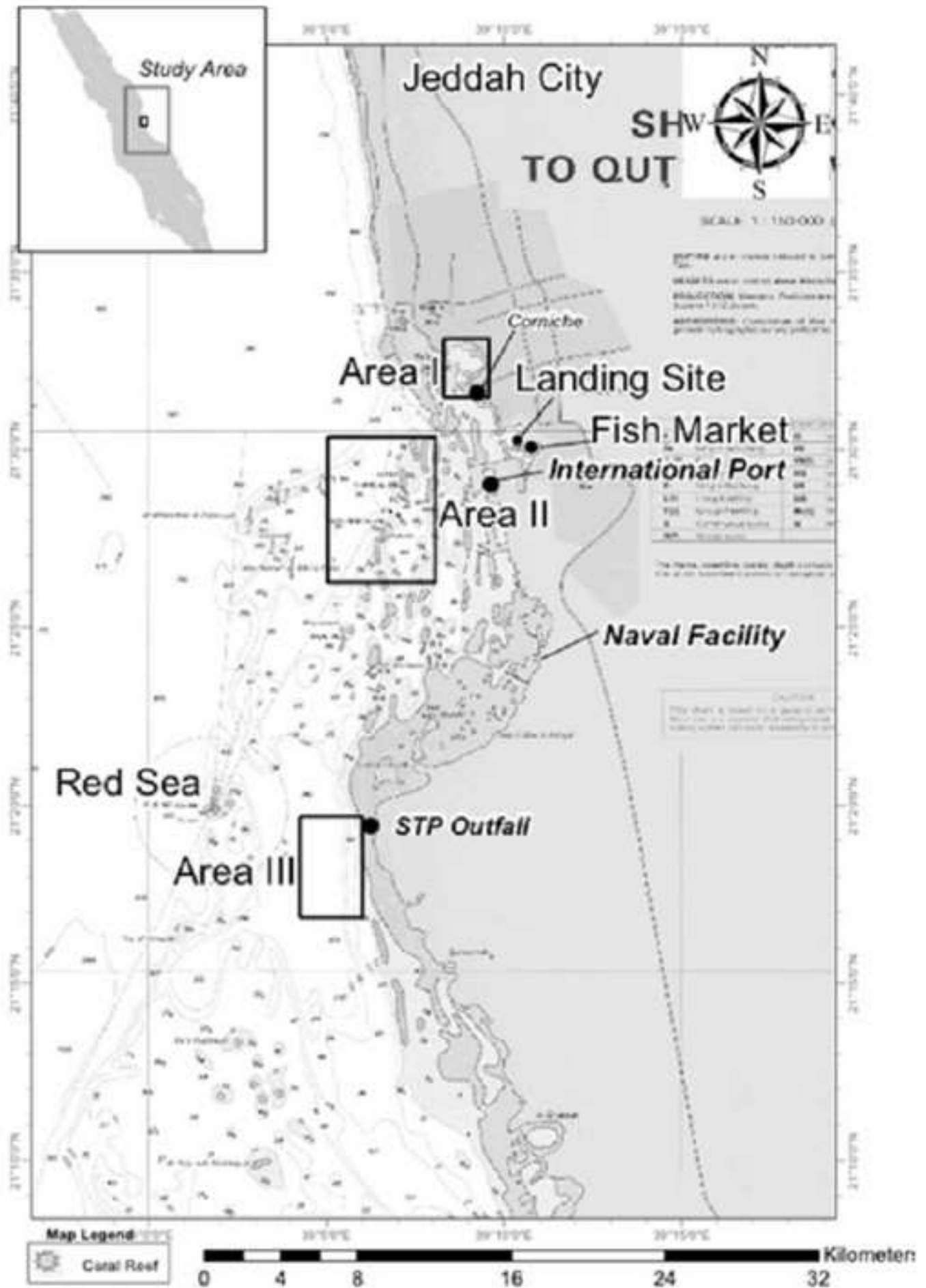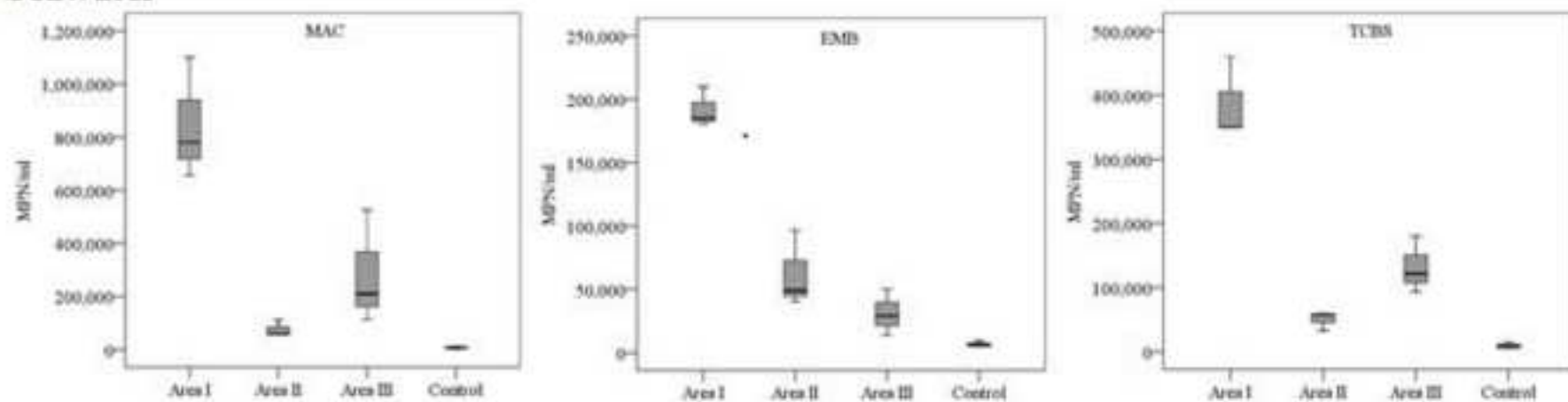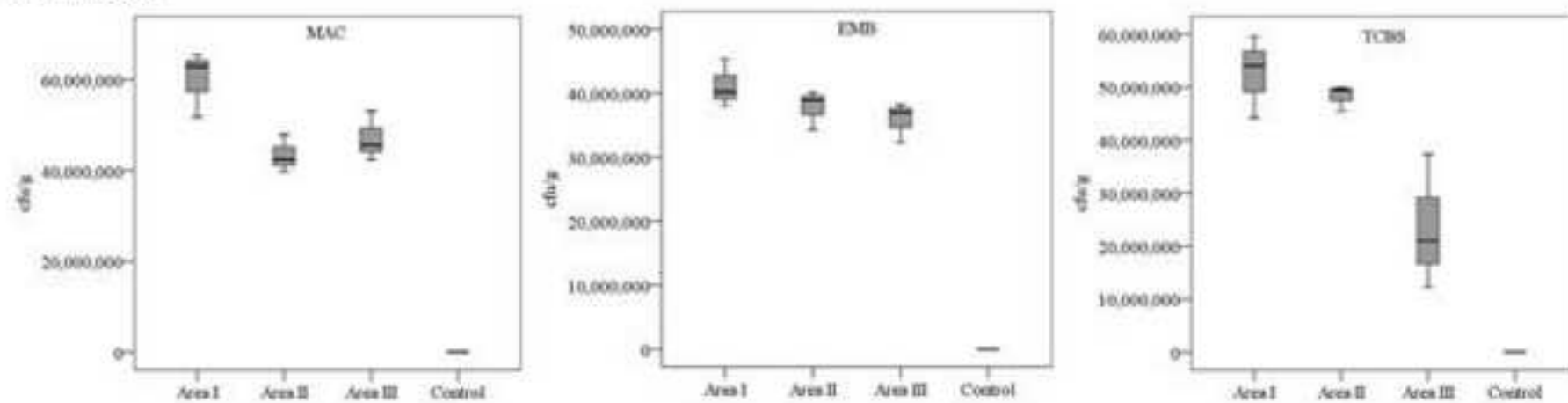
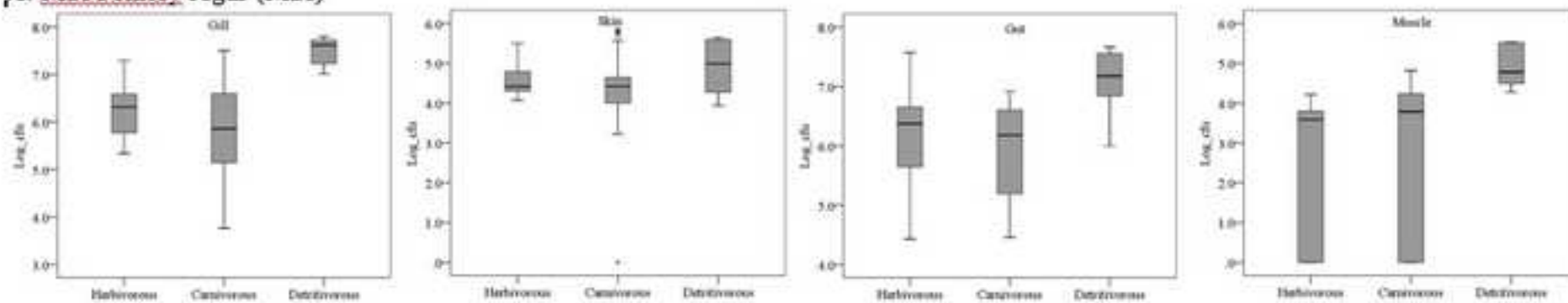## A. MacConkey Agar (Mac)
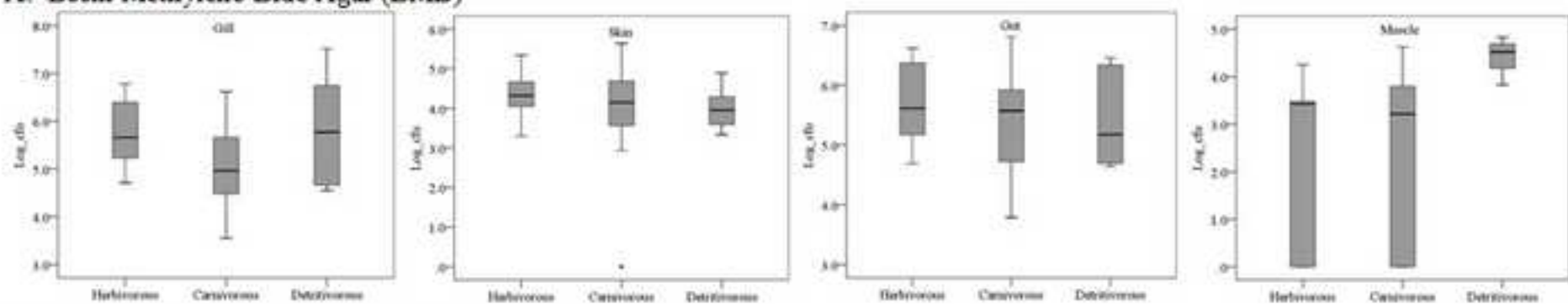

B. Eosin Methylene Blue Agar (EMB)

C. Thiosulfate-Citrate-Bile Salts agar (TCBS)

Figure 7

A. MacConkey Agar (Mac)

A. Eosin Methylene Blue Agar (EMB)

B. Thiosulfate-Citrate-Bile Salts agar (TCBS)

Figure 8