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BACTERIOLOGICAL QUALITY AND METAL CONTENTS OF *DIPLODUS VULJARIS* AND *SIGANUS RIVULOTUS* IN THE EASTERN HARBOUR WATER: A COMPARATIVE STUDY OF FRESHLY HARVESTED AND MARKET FISH

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ABSTRACT

A diverse array of bacterial species including several potential human pathogens, were isolated from *Diplodus vuljaris* and *Siganus rivulotus* harvested from the Eastern harbour water. High numbers of fecal indicator bacteria (*E. coli* and *S. faecalis*) were detected as well as species of *Vibiro, Salmonella, Staphylococcus* and *Aeromonas*. Muscle tissues contained much lower levels of bacteria than other tissues. In general, samples collected from the market contained higher levels of bacteria compared to fresh samples. Siganus rivulotus harboured the highest numbers of fecal indicators and pathogenic bacteria compared to Diplodus vuljaris. The fish was also contaminated with considerable amounts of heavy metals (Cd, Cu, Pb, Zn and Ni). The data confirm the necessaty for waste water treatment before discharge into the harbour for environmental safety.

INTRODUCTION

Water and fish quality is a very important subject to protect public health with a special emphasis on the microbiology and heavy metal concentrations. The high levels of pollution and domestic sewage increase the persistence of fecal indicators bacteria and other pathogens. Human pathogens have been reported to be associated with different types of fish (Rivera *et al*, 1999; Gonzalez. *et al*, 1999; Garcia Lopez *et al*, 2004).

Fish living in polluted waters may accumulate toxic trace elements via their food chains. Heavy metals have an affect on the physiology and metabolism of fish (Heba, 1992). Trace metals also have an effect on the growth and protein turnover of fishes from tropical and temperate areas (Hassan and Nadia, 2000). Many investigations for the environmental conditions and levels of heavy metals in fish have been carried out in Alexandria by several workers (Abul dahab *et al*, 1990; Yusef *et al*, 1995; El-Rayis *et al*, 1997 and Siam, 2003).

The present work is devoted to investigate the bacterialogical quality of names with a special emphasis on coliforms and some human pathogens. The level of some metals (Cd, Cu, Pb, Zn and Ni) in muscle tissues and different organs of fish was evaluated. A comparison was done between freshly harvested fish and those taken from the market.

MATERIALS AND METHODS

Study area: The Eastern harbour is a shallow, protected embayment, semi enclosed circular basin covering an area of about 2.8 km^2 and occupying the central part of the coast of Alexandria. It receives a daily amount of about 63.000 cubic meters of unprocessed sewage which affects the

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physical, chemical and biological characteristics of harbour waters. Eleven outfalls discharging untreated sewage into the embayment exist inside the harbour (Said and Maiyza, 1987).

Water sample collection: The bacteriological sampling techniques of the International Organization for Standardization ISO 5667/1 (1980) and ISO 5667/2 (1990) were used for water sample collection. Sterile glass sampling bottles with wide-mouth and screw caps with capacity of 500 ml were used for collecting water samples. Special stainless steel sampling rod was used.Samples were collected about 25-35 cm below water surface, the bottles were kept unopened until the moment of collection. Samples for the physico-chemical analysis were fixed in position immediately after taking them. After collection the samples were sent to the laboratory, and examined within two or three hours of sampling. Two separate samples were collected from the same source at the same time.

Fish samples: Samples were collected during April, 2002. Fresh fish were caught from the Eastern harbour area. Other samples were collected from the market of the same area, then placed in plastic bags. Fish were transported to the laboratory in an ice box. Those choosen for this study had average length from 11.7 to 12.2 cm and average weight from 36.5 to 38 gm. Upon arrival, organs of gills, brain, ovary and muscles were separated. One gm of each organ was placed in sterile container containing sterile saline solution (1% Na Cl) as diluent. Each sample was blended for 5 min. three samples from each type were examined.

Media: Media used for detection and confirmatory tests, were purchased from Difco laboratories, Detroit, Mich, and prepared according to the manufacturer's instructions.

Bacterial count: For enumerating total viable bacteria, serial dilutions (10^{-2} to 10^{-5}) of the water sample and the homogenates were prepared in four sterile test tubes, each containing 9 ml of sterile 1% Na Cl solution. The viable bacterial count in each sample was determined by mixing one ml from each dilution with 20ml molten sterile nutrient agar (45^{o}C), poured in petri-plates, shaked well and left until solidification. All inoculations were performed in triplicate. Petri-dishes were incubated at 28^{o}C for 48h. then developed colonies were counted.

Bacteriological procedures: Bacterial analyses were performed by the membrance filtration technique according to ISO 9308/1 (1990) and 7899/2 (1984). Quantities of 0.1, 1 and 10 ml of each sample were filtered through 0.45 µm pore size 47 mm diameter, gride sterile cellulose membrane (Gelman Laboratory) using a sterile glass filtration unit (Millipore, Befrid, UK) and a vaccum pump at a pressure of 65 k Pa.For enumerating total coliforms, the membranes were placed onto the surface of Endo-LES agar and incubated at 37^{o}C ± 0.5 for 24h. The dark red colour with golden-green metallic sheen colonies on the used selective medium were counted. Representative of ten colonies were subcultured onto nutrient agar (37^{o}C for 24h) and the confirmatory tests including gas production (using lactose broth medium), oxidase test and Gram-stain were done. For detection and counting the thermotolerant coliforms (pres *E. coli*), the membranes were placed onto the surface of m-FC agar and incubated at 44.5^{o}C ± 0.5 for 24h. The blue colonies developed, were counted and ten colonies were selected and subcultured on nutrient agar (N.A.) (at 37^{o}C for 24h). Confirmatory tests including gas production, indole production, oxidase test and Gram-stain were performed for the choosen isolates.

For investigation and counting of *Streptococcus faecalis*, the membranes were placed onto the surface of m-enterococcus agar and incubated at 37^{o}C for 48h. Red, maroon or pink colonies were counted, then subcultured on N.A. at 37^{o}C for 24h. For the ten selected colonies, confirmatory tests including esculin hydrolysis, catalase test and Gram-stain were done.

For detection of *Vibrio spp.*, the membranes were placed onto the surface of alkaline peptone agar for 6 h. at 25°C then carefully transferred onto TCBS agar and incubated at 37°C for 24h. Large green and/or yellow colonies are characteristic for *Vibrio spp.*

For the isolation of *Aeromonas spp.*, the membranes were placed onto the surface of *Aeromonas* Medium Base (Ryan) (CMO833) with Ampicillin selective supplement (SRO136) for 24h. at 37°C. Yellow colonies developed represent *Aeromonas spp.*

Staphylococci were isolated by placing the membranes onto the surface of mannitol salt agar for 24h. at 37°C which developed as yellow colonies.

Detection and Enumeration of *Salmonella:* This was performed in 3 steps:

1. Pre-enrichment in non-selective liquid medium: where buffered peptone water was inoculated with the test sample, and incubated at 37°C for 16 to 20h, was carried out.
2. Enrichment in selective liquid media. Magnesium chloride/malachite green and selenite/cystine media were inoculated with cultures obtained in 1. Magnesium chloride/malachite green medium was incubated at 42°C for 24h. whereas selenite/cystine medium was incubated at 37°C for 24h.
3. Plating out and recognition. Cultures obtained in 2, were transferred into a selective solid medium of phenol red/brilliant green agar. Plates were incubated at 37°C and examinated after 24h. and if necessary, after 48h. to check for the presence of typical colonies of Salmonella. Typical *Salmonella* colonies caused change to the phenol red/brilliant green agar from pink to red. Confirmatory tests were performed as describe d in APHA (1989).Counts of all bacterial groups are given as CFU/gm.

Determination of heavy metals in fish organs:
The analyses were carried out according to UNEP/FAO/IAEA/IOC (1984). In this procedure, an exact weight of wet sample (not more than 1g) was placed in teflon vessels and 4ml of analar nitric acid was added to soft organs. The vessels were covered and placed in a steel block which was closed tightly. Samples were allowed to predigest at room temperature over night. The digestion block was placed on a preheated hot plate at 100°C for two hours. The samples were cooled to room temperature and transfered to 25ml volumetric flasks. If the solution was not clear, it was reheated for another 60 min. at 100°C. The metals Cd, Cu, Pb, Zn and Ni were determined by atomic absorption spectrophotometer. The results were expressed in μg/g (ppm).

RESULTS

Physical properties and bacterial count in sea water: Data in Table 1. show the presence of high bacterial counts (29 x 10^2/ml). Total coliforms showed the highest count (5 x 10^2/ml) compared to other bacterial groups.

Enumeration of total viable bacteria associated with fish tissues: The results in Table 2. indicate that the gills harboured higher counts than other fish organs, whereas muscles contained the least count. Moreover, in general the bacterial content in fish organs obtained from market was much higher than those found in fresh caught fish.

Fecal indicator bacteria in fish organs: In most cases, *S. rivulotus* samples, had greater concentrations of fecal indicators than D. vuljaris samples, (Table 3). For example in the market samples of *D. vuljaris* fecal indicators were absent in all organs except in gills.

The gills harboured the highest concentrations of faecal indicators while none was detected in muscles (data not shown). Fresh samples of S. rivulotus contained 620 CFU/gm of *E. coli* and 1280 CFU/gm of *S. faecalis.*

In most organs, the market samples were lower in count compared to fresh samples. For example, the count of S. faecalis (1280 CFU/gm gills) of fresh S. rivulotus was almost 32 times higher than the count in the market samples (40 CFU/gm gill)

On the other hand, gills of *D. vuljaris* collected from the market contained higher counts than those freshly harvested.

Although *E. coli*, is the widely used indicator of fecal contamination, the Gram-negative bacterium *S. faecalis* was more prevalent.

Table 1. Physical,chemical and bacteriological characteristics of the Eastern Harbour water during spring, 2002.

Physico chemical characteristics	**Value**
Temperature (^oC)	23.50
PH	8.20
Alkalinity (ml eq./L)	3.33
Salinity ($S\%_o$)	36.91
Dissolved oxygen (ml O_2/L)	3.12
Oxidizable organic matter (mg O_2/L)	6.10
Total nitrogen (µg at./L)	11.82
Dissolved nitrate (µg at. NO_3-N/L)	0.39
Dissolved nitrite (µg at. NO_2-N/L)	6.53
Dissolved ammonia (µg at. NH_3-N/L)	4.90
Reactive phosphate (µg at. PO_4-P/L)	1.37
Bacterial count	CFU/100ml
Total viable count	290000
Total coliforms	50000
Streptococcus faecalis	9600
Escherichia coli	5100
Vibrio spp.	2000
Salmonella spp.	400
Staphylococcus spp.	1200
Aeromonas spp.	200

Table 2: Viable bacterial count associated with D. vuljaris and S. rivulotus organs.

Species	**Count (CFU/gm)**			
	Ovary	**Brain**	**Gills**	**Muscles**
Diplodus vuljaris (Fresh)	28×10^3	2×10^5	75×10^5	22×10^3
Diplodus vuljaris (Market)	2×10^5	32×10^4	8×10^7	25×10^3
Siganus rivulotus (Fresh)	254×10^3	206×10^3	964×10^5	37×10^3
Siganus rivulotus (Market)	6×10^5	8×10^5	12×10^7	55×10^3

Other pathogenic bacteria in fish organs: The data in Table 4. show that the prevalence of species of *Vibrio, Salmonella, Staphylococcus* and *Aeromonas* in all organs of D. vuljaris and *S. rivulotus* (fresh and market samples) except for the muscles which had no pathogens (data not shown).

The gills were the most contaminated organ, they harboured 18400,6000 and 6000 CFU/gm) of *Vibrios, Staphylococcus* and *Aeromonas* spp. respectively in market samples of *S. rivulotus.*

With few exceptions, the fresh samples were lower in the count than the market samples for example, the count of *Staphylococcus* spp. and *Aeromonas* spp. in the gills of *S. rivulotus* (market samples) were 6000 CFU/gm while in (fresh samples) were 1640 and 630 CFU/gm respectively.

Concentration of heavy metals in fish organs:

Cadmium: The concentration of cadmium in *D. vuljaris* and *S. rivulotus* organs ranged from 0.29 to 16.88 ppm. The highest concentrations were found in the gills and muscles of *D. vuljaris* (market) (16.88 and 14.48 ppm) respectively, the toxicological tolerance level is (1ppm) and we can conclude that all the gills samples and the muscles only of *D. vuljaris* were highly contaminated.

Cupper: D. vuljaris contained the highest levels of cupper especially in the muscles and the brains (4.77 ppm) but the toxicological tolerance level is (20 ppm) so that all concentrations in the different organs lie in the normal range.

Lead: Brain and gills of S. rivulotus (market) and the ovary of *D. vuljaris* (market) are considerably contaminated with this metal (6.76 ppm).

Zinc: In our study, there is a highly significant difference between the concentrations of Zn^{2+} in the different organs where low concentrations were detected in the brains of both fish (1.91-3.59 ppm .Then highest concentrations (31.79 and 192.63 ppm) were recorded in gills of fresh and marked samples of *S. rivulotus.*

Nickel: The toxicological tolerance level of nickel is (55 ppm) and our readings did not exceed that limit as the highest concentration recorded was (27.79 ppm) in the gills of *D. vuljaris* (market).

The cadmium and lead concentrations exhibit a serious status due to their high levels in the different organs and the market samples were more polluted than the fresh samples.

DISCUSSION

The Eastern harbour water recieves huge amount of discharged sewage (Said and Maiyza, 1987) and that could be considered the reason for the presence of these high counts of the investigated bacteria in the harbour. It is well known that the microbiological quality of fishes is directly related to the quality of the water in which they are cultivated and/or harvested, the presence of fecal indicators bacteria in the water could be correlated with the presence of some other pathogens (El-Shenawy *et al,* 2000). However, some investigators have shown that no correlation was found between the presence of fecal coliforms and other some pathogens (Kaper *et al,* 1979; Colwell *et al,* 1981). Some of the human pathogens can survive and some can grow at low temperatures. Rivera et al (1999) on the other hand concluded that higher water temperatures apparently enhance bacterial proliferation in the haemolymph of living crabs.

Table 3 : Fecal indicator bacteria associated with *D. vuljaris* and *S. rivulotus* organs.

Species	**Count (CFU/gm)**								
	Ovary			Brain			Gills		
	Total coliforms	*E. coli*	*S. faecalis*	Total coliforms	*E. coli*	*S. faecalis*	Total coliforms	*E. coli*	*S. faecalis*
D. vuljaris (Fresh)	2000	0	80	2000	0	720	1840	0	320
D. vuljaris (Market)	0	0	0	0	0	0	4000	480	720
S. rivulotus (Fresh)	3400	480	560	4000	0	320	4200	620	1280
S. rivulotus (Market)	840	60	60	420	80	80	4000	40	40

Table 4. Count of some pathogenic bacteria associated with *D. vuljaris* and *S. rivulotus* organs

Organ / Species	Count (CFU/gm)											
	Ovary				Brain				Gills			
	Vibrio	*Salmonella*	*Staphy lococcus*	*Aeromonas*	*Vlbrio*	*Salmonella*	*Staphy lococcus*	*Aeromonas*	*Vlbrio*	*Salmonella*	*Staphy lococcus*	*Aeromonas*
D. vuljaris (Fresh)	640	0	400	280	800	800	560	200	2400	200	860	400
D. vuljaris (Market)	860	460	0	560	4000	400	800	500	4400	1600	2200	1200
S. rivulotus (Fresh)	2400	440	840	400	800	40	560	350	4600	1200	1640	630
S. rivulotus (Market)	4200	160	2640	1400	4240	840	1200	900	18400	600	6000	6000

In Egypt, there are no federal microbiological standards for fish because the indicator organisms present on fish caught from polluted waters which are not generally considered part of the normal flora of the fish or their environment (Matches & Abetyla, 1983). Thus, the data of the present study show that fecal bacteria could be considered as a localized contamination from water where the fish were cultivated and/or harvested. In this study, the bacterial burdens of most organs were moderate and in the range reported in the literature (Tubias *et al.,* 1975; Sizemore *et al,* 1975).

It has been found that *S. rivulotus* were more contaminated than *D. vuljaris* in all samples and that might be due to the slimy nature of the skin surface and the lack of small scales which covers the Diplodus surface (FAO, 1973).

None of the fecal indicator bacteria or the other pathogens were detected in fish muscles while the gills harboured the highest numbers and that agreed with previous data (Faghri *et al*, 1984; El-Shenawy *et al*, 2000).

Samples from the market harboured higher numbers than the fresh samples due to the fact that death of fish leads to increase of the numbers significantly. This is in accordance with data published by Faghri *et al,* (1984), but in most samples, the numbers of fecal indicators of market samples were lower than those of fresh samples and that agrees with Dutka (1973) and Rhodes *et al*, (1983) who reported that many studies have shown the inadequency of standard coliform counts as an indicator of fecal contamination of marine ecosystems and the safety of shelfish collected from areas impacted by sewage effluents .*E. coli* is rapidly eliminated from seawater, whereas other bacteria in sewage effluents, including human pathogens, may survive for longer periods of time.

Vibrio spp. were the predominant bacterial pathogens and that agreed with Rivera *et al* , (1999) in his study on crab haemolymp. In all infected organs, however, their percentages did not exceed 1% of the total bacterial flora (184 x 10^2 CFU/gm). Davis and Sizemore (1982) found that the *Vibrio spp.* levels in the stomach and gills of *Callinectes sapidus* routinely reached concentrations greater than 10^8 CFU/gm and these levels are sufficient to constitute an infective dose if even 1% are pathogens.

A serious state is the incidence of enteric pathogen *Salmonella spp.* in the Eastern harbour water and its spread in all samples in spite of their low percentage (0.2%). This refers to the necessity of treating waste water before discharging into the harbour.

It was also noticed that *Stapylococcys spp.* and *Aeromonas spp.* are vigorously spread in the gills and with less extent in the ovary and the brain samples and their absence in the muscles. On the contrary Faghri et al, (1984) reported that *Stapylococcus* species were abundant in the muscle and gill tissues of all crabs. Also, Rivera *et al,* (1999) isolated *Aeromonas hydrophyla* and *A. salmonicida* from the crab haemalymph.

The high density and diversity of bacteria in the different fishes organs, the ingestion of uncooked *D. vuljaris* and *S. rivulotus* may represent a potential threat to human health.

The study revealed that in all tested fish samples, accumulation of metals (Cd, Cu, Pb, Zn and Ni). In general, muscles was lower in metal content compared to other organs. Simiral observations were found in other studies (Vigh *et al,* 1996; Hassan and Nadia; 2000 and Siam, 2003). The content of each metal in a specific organ differed according to metal ion and type of fish. Variation in metal contents in fish organs was reported by others (Emara *et al,* 1993; Matta *et al,* 1999; Zaube *et al,* 1999). This variation is dependent on type of fish and its source. In our study, metal content was similar in freshly harvested fish and market fish did not exceed the toxicological level except for lead where levels higher than 5 ppm were recorded.

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