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Full Length Research Paper

Effect of water pollution on expression of immune response genes of *Solea aegyptiaca* in Lake Qarun

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This research was aimed to study quality of water in Lake Qarun and effects of pollution on expression of immune genes in Egyptian sole (*Solea aegyptiaca*) fish. The study was carried out from August 2006 to the end of April 2007. The water samples were collected from different locations of Lake Qarun at Al-Oberge within an area of 200 to 1500 meter from the shore. The samples were subjected to different physical and chemical analyses. The concentration of total dissolved solids (TDS) recorded an average value of 37.8 g/l while the chloride content was 14.3 g/l on average. The corresponding value of salinity was 25.9 g/l. For the chemical oxygen demand and biochemical oxygen demand the results revealed that the average values were 98 ± 22 mg O_2/l and 8.0 ± 2.1 mg O_2/l for the chemical oxygen demand (COD) and biochemical oxygen demand (BOD), respectively. Analyses of nitrogen group indicated presence of low concentrations of all. For total pesticides which might be one of the most potential pollutants in the lake, the results showed an average value of 0.62 mg/l. Furthermore, the total viable bacterial count (TVBC) ranged from 10^3 colony forming unit (CFU) in the middle of the Lake to 10^7 (CFU) near the shore. Stressed total coliform group (STC), stressed faecal coliform group (SFC) and stressed faecal streptococci group (SFS) increased from the middle of the Lake to the shore. The differential expression of the immune genes, that is, GARP and SIMP genes, as a result of pollution influence was further confirmed by RT-PCR, with the up-regulation of these genes in the liver of the collected fish. The application of the expression of the immune genes of fish might be time safe and cost effective in case there are different source of pollution.

Key words: Pollution, Lake Qarun, *Solea aegyptiaca*, immune genes.

INTRODUCTION

Pollution of the aquatic environment is a serious and growing problem (Sasaki et al., 1997). Increasing number and amount of industrial, agricultural and commercial chemicals discharged into the aquatic environment having led to various deleterious effects on the aquatic organisms (McGlashan and Hughies, 2001). Aquatic organisms, such as fish, accumulate pollutants directly from contaminated water and indirectly via the food chain (Sasaki et al., 1997).

Heavy metals toxicity has been extensively studied in fish (Chan et al., 1999; Heing and Tate, 1997). Application of chemical fertilizers containing trace of heavy metals causes contamination of fish with these metals (Chaisemartin, 1983). The effects of pesticides either organophosphorous or chlorinated pesticides have been extensively studied and confirmed in fish (Rao, 2006a; Rao, 2006b; Pandcy et al., 2006; Capkin et al., 2006).

In Egypt, fisheries and aquaculture is an important component of the agriculture sector and represents a significant source of animal protein. It represented 4% of agriculture production and 14% of the total livestock and poultry production by value in 1994 (Shehadeh and Feidi, 1996). The major part of Egyptian fish production (capture and aquaculture) is coming from inland fish production, where Egypt is ranked as 7th of the top ten countries in inland fish production (FAO, 2000). Fish production in brackish water is the main source and only

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453 ton was harvested from marine water.

Lake Qarun is brackish water lake which was originated from a fresh water lake called Moreis. It is a closed system seed as a reservoir for agricultural drainage water of Fayoum Province. Extensive evaporation of water from such closed ecosystem increases concentration of salts, heavy metals, pesticides and other pollutants. Consequently, this changes the quality of water and affects biology of lake. A number of investigations have concerned the water chemistry (Naguib, 1958; Bishai and Kirollus, 1980; Mansour et al., 2000) and microbiology (Mansour and Sidky, 2003) of Lake Qarun.

The impact of toxic materials on the integrity and functioning of DNA has been investigated in many organisms under field conditions (Bombail et al., 2001). Several biomarkers have been utilized as tools for detection of exposure to genotoxic pollutants. Such biomarkers include presence of DNA adducts, chromosomal aberrations, DNA strand breaks and micronuclei measurements. In fish, erythrocytes are mainly used as sentinel markers of exposure to genotoxic compounds (Mitchelmore and Chipman, 1998; Bombail et al., 2001; Gontijo et al., 2003).

Analysis of gene expression is increasingly important tool in many research fields (Mocellin et al., 2003; Koyanagi et al., 2005). Reverse transcription polymerase chain reaction (RT-PCR) is established method commonly used to measure transcript abundance in biological samples. RT-PCR permits the simultaneous analysis of the expression levels of a small number of genes in many different samples (Ishii et al., 2007). Despite the limitation in the number of genes that can be analyzed per sample, RT-PCR has two key advantages, namely high detection sensitivity with wide dynamic range and quantitative data generation with good reproducibility (Ishii et al., 2007).

To evaluate the pollution possibilities in Lake Qarun, Semi-quantitative RT-PCR was carried out to examine the changes in the transcripts levels of several immune genes of Egyptian sole (*Solea aegyptiaca*) fish in relation to the water quality in the Lake

MATERIALS AND METHODS

Physicochemical analysis of water

The samples of water were collected from Lake Qarun at Al-Oberge within an area of 200-1500 meter from the shore. The study was carried out from the beginning of August 2006 to the end of April 2007. The samples for physicochemical analyses were collected in 4-liter plastic bottles and preserved in an icebox. The samples were transported to the lab within one and half hour for the analysis of chemical oxygen demand (COD), biochemical oxygen demand (BOD), total dissolved solids (TDS), chloride content, salinity, total hardness, alkalinity, ammonia nitrogen, nitrite nitrogen and nitrate nitrogen. Temperature and pH was measured in the field using portable pH meter model Jenway. Dissolved oxygen (DO) was preserved in the field and measured in the lab using BOD bottle and Azid modification method (APHA, 1998).

COD was analyzed colorimetrically according to APHA (1998) by using NANOCOLOR Linus spectrophotometer. Biochemical oxygen demand was analyzed according to the standard methods (APHA, 1998) using dilution method. Total dissolved solids were measured after filtration through membrane filter with 0.45 µm. Chloride content was measured according to APHA (1998) using argentomertic method. Ammonia was measured using phenate methods while nitrite was measured using azodye method (APHA, 1998). Nitrate was measured using sodium salicylate method. Total pesticides were analyzed using HPLC.

Microbiological examination

The samples for microbiological examination were collected from the study area. Five samples were collected at the study area between 200 to 1500 meter from the shore (200 m, 500 m, 800 m, 1100 m and 1500 m). Another sample (sample number 6) was collected from agriculture drains feeding the lake at the water inlet point. Total viable bacterial count (TVBC), stressed total colifom (STC), stressed faecal colifom (SFC) and stressed faecal streptococci (SFS) were determined according to the standard method (APHA, 1998).

Semi-quantitative RT-PCR

Fish Sampling

During the period of August 2006 to the end of April 2007, fresh samples of Egyptian sole (*S. aegyptiaca*) fish were caught from the studied area. In addition, control fish samples of Egyptian sole were purchased from a private fish farm located in Giza governorate, Egypt. The samples were transferred quickly, in an ice-box to the laboratory.

RNA extraction

In the laboratory, liver samples were taken from 6 fish of Egyptian sole (*S. aegyptiaca*) fish. The samples were immediately frozen in liquid nitrogen and stored at −80°C prior to extraction. Total RNA was isolated from 50 to 100 µg of liver tissue by the standard TRIzol extraction method (Invitrogen, Paisley, UK) and recovered in 100 µl molecular biology grade water. In order to remove any possible genomic DNA contamination, the total RNA samples were pretreated using DNA-free™ DNase treatment and removal reagents kit (Ambion, Austin, TX, USA) following the manufacturer's protocol.

Reverse transcription

The complete Poly(A)+ RNA isolated fish samples was reversely transcribed into cDNA in a total volume of 20 µl using 1 µl oligo(dT) primer. The composition of the reaction mixture, termed as master mix (MM), consisted of 50 mM MgCl2, 10x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; Perkin-Elmer), 10 mM of each dNTP (Amersham, Brunswick, Germany), and 50 µM of oligo(dT) primer. The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and finished with denaturation step at 99°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through polymerase chain reaction (PCR).

Table 1. Primers and reaction parameters in RT-PCR.

Target cDNA	Primer name	Primer sequence (5′–3′)	Annealing temperature (°C)	PCR product size (bp)
β-Actin	Act-F Act-R	CCCCATCGAGCACGGTATTG ATGGCGGGGGTGTTGAAGGTC	57	189
SIMP	SIMP-F SIMP-R	ACCCCAGTCCCAGCGTAGTG ATGTCGTCGCCTGAGTATCCAATC	58	331
GARP	GARP-F GARP-R	CCTTCACGGCAACAACATCC ATCACGCTAATCCACAACAC	54	1,656

Table 2. Physicochemical characteristics of water samples collected from Lake Qarun.

Parameter	Unit	Minimum	Maximum	Average±SD
Temperature	°C	25	31	-
pH	-	8.3	8.8	-
Dissolved oxygen	mg O_2/l	6.5	10.2	8.5±1.3
Chemical oxygen demand	mg O_2/l	65	122	98±22
Biochemical oxygen demand	mg O_2/l	4.3	10.2	8.0±2.1
Total dissolved solids	g/l	30.3	45.9	37.8±45.9
Chloride	g/l	13.7	15.0	14.3±0.35
Salinity	g/l	24.8	27	25.9±0.6
Total hardness	mg/l as CaCO3	6090	6322	6191±89
Total alkalinity	mg/l	148	170	156±7
Ammonia nitrogen	mgN/l	0.12	0.52	0.34±0.11
Nitrite nitrogen	mgN/l	0.008	0.11	0.021±0.030
Nitrate nitrogen	mgN/l	0.085	0.13	0.109±0.013
Total pesticides	mg/l	0.44	0.73	0.62±0.12

Polymerase chain reaction (PCR)

The first strand cDNA from different fish samples was used as templates for RT-PCR with a pair of specific primer. The sequences of specific primer and product sizes are listed in Table 1. β-Actin was used as a housekeeping gene for normalizing mRNA levels of the target genes. The reaction mixture for RT-PCR was consisted of 10 mM dNTP's, 50 mM MgCl2, 10x PCR buffer (50 mM KCl; 20 mM Tris-HCl; pH 8.3; Gibco BRL, Eggenstein, Germany), and auto-claved water. The PCR cycling parameters were one cycle of 94°C for 3 min, 35 cycles of 94°C for 30 s, 42°C to 58°C for 30 s, 72°C for 90 s, and a final cycle of 72°C for 7 min. The PCR products were then loaded onto 2.0% agarose gel, with PCR products derived from β-actin of the different fish samples.

RESULTS

Physicochemical analysis of water

Physicochemical characteristics of water in the study area are presented in Table 2. The results showed that the concentration of total dissolved solids (TDS) ranged from a minimum value of 30.3 g/l to a maximum value of 45.9 g/l with an average value of 37.8 g/l. In the same manner the chloride content ranged between 13.7 and 15.0 g/l with an average value of 14.3 g/l. The corresponding values of the salinity were 24.8 g/l, 27 g/l and 25.9 g/l for the minimum, maximum and average value, respectively.

For the COD and BOD, the results revealed that the average values were 98 ± 22 mg O_2/l for the COD and 8.0 ± 2.1 mg O_2/l for the BOD. For the nitrogen group the analysis of ammonia nitrogen, nitrite nitrogen and nitrate nitrogen indicated presence of low concentrations of all. In this study the ammonia concentrations ranged between 0.12 to 0.52 mg N/l with 0.34 mg N/l as average value. The nitrite concentrations recorded an average value of 0.03 mg N/l and a maximum value of 0.11 mg N/l. For total pesticides the results showed an average value of 0.62 mg/l.

Table 3. Microbiological quality of water samples collected from Lake Qarun.

Sample	1	2	3	4	5	6
Total viable bacterial count (cfu/ml at 37°C)						
Minimum	$1.2 \cdot 10^3$	$2.2 \cdot 10^3$	$3.2 \cdot 10^3$	$2.2 \cdot 10^4$	$2.2 \cdot 10^5$	$1.1 \cdot 10^6$
Maximum	$7.7 \cdot 10^4$	$3.3 \cdot 10^4$	$9.0 \cdot 10^4$	$4.6 \cdot 10^6$	$6.4 \cdot 10^6$	$4.8 \cdot 10^7$
Average	$2.1 \cdot 10^4$	$1.2 \cdot 10^4$	$4.8 \cdot 10^4$	$1.2 \cdot 10^6$	$3.4 \cdot 10^6$	$2.1 \cdot 10^7$
Stressed total coliform (cfu/100 ml)						
Minimum	$3.0 \cdot 10$	$3.0 \cdot 10$	$1.2 \cdot 10^2$	$1.4 \cdot 10^2$	$1.3 \cdot 10^2$	$2.2 \cdot 10^4$
Maximum	$1.8 \cdot 10^2$	$3.3 \cdot 10^2$	$4.4 \cdot 10^2$	$4.9 \cdot 10^2$	$9.3 \cdot 10^2$	$8.4 \cdot 10^5$
Average	$8.4 \cdot 10$	$1.7 \cdot 10^2$	$2.2 \cdot 10^2$	$2.9 \cdot 10^2$	$3.9 \cdot 10^2$	$2.7 \cdot 10^5$
Stressed faecal coliform (cfu/100 ml)						
Minimum	$1.1 \cdot 10^1$	$2.2 \cdot 10^1$	$2.0 \cdot 10^1$	$3.0 \cdot 10^1$	$6.0 \cdot 10^1$	$1.1 \cdot 10^3$
Maximum	$6.5 \cdot 10^1$	$8.0 \cdot 10^1$	$2.0 \cdot 10^2$	$2.2 \cdot 10^2$	$4.8 \cdot 10^2$	$5.5 \cdot 10^4$
Average	$2.8 \cdot 10^1$	$5.1 \cdot 10^1$	$7.5 \cdot 10^1$	$1.2 \cdot 10^2$	$2.1 \cdot 10^2$	$1.6 \cdot 10^4$
Stressed faecal streptococci (cfu/100 ml)						
Minimum	$1.0 \cdot 10^1$	$2.0 \cdot 10^1$	$1.0 \cdot 10^1$	$2.0 \cdot 10^1$	$3.0 \cdot 10^1$	$2.0 \cdot 10^3$
Maximum	$1.3 \cdot 10^1$	$5.0 \cdot 10^1$	$6.0 \cdot 10^1$	$1.4 \cdot 10^2$	$2.5 \cdot 10^3$	$4.5 \cdot 10^4$
Average	$1.2 \cdot 10^1$	$2.9 \cdot 10^1$	$3.0 \cdot 10^1$	$7.3 \cdot 10^1$	$7.2 \cdot 10^2$	$1.3 \cdot 10^4$

Microbiological examination

The results of the microbiological quality of water samples are presented in Table 3. In the middle of the Lake, TVBC fluctuated around 10^3 and 10^4 CFU/ml. Near the shore, TVBC reached 10^6 CFU/ml. STC decreased from 10^2 CFU/100 ml near the shore to 10^1 CFU/100 ml in the middle of the Lake. The same decrease was observed in case SFC and SFS. In addition, some samples were free from SFS. The inlet water showed higher bacterial count than the lake water.

Semi-quantitative RT-PCR

In order to confirm that the presence of pollution, RT-PCR assay was conducted to verify the expression of immune genes in the liver of Egyptian sole (*S. aegyptiaca*) fish collected from the study area in Lake Qarun: compared with control fish collected from private fish farm. GARP gene and SIM gene were up-regulated in all samples of Egyptian sole (*S. aegyptiaca*) fish (Figures 1 and 2). However, the expression of GARP and SIMP genes was down-regulated in the control Egyptian sole fish.

DISCUSSION

In the present study, values of dissolved solids are higher than the values measured by Mansour and Sidky (Mansour and Sidky, 2003). The values of chloride are higher than what has been recorded between 1997-1999 (Mansour and Sidky, 2003). The variation in the results could be attributed to two main reasons. First is the variation in the sampling site, which is affected by distance from the water inlet points in the Lake and the wastewater discharge points from the restaurants and cafeteria along the Lake. In this study the water samples have been collected from the area near the mouth which is little far from any water inlet points. The second reason is the time gap since the Lake is closed and the salt content might increase by time. The variation in the concentration of TDS and salinity is mostly because of the variation in the sampling points. The samples near to the shore are highly affected by the discharge of wastewater from the drains and the restaurants along the beach.

The results for the chemical oxygen demand and biochemical oxygen demand indicated presence of non-biodegradable source of pollution, which might be micro-pollutants in nature or reduced form of salts. The level of total pesticides recorded 0.6 mg/l in average. Many studies have proven the carcinogenicity and genotoxicity of pesticides specially the chlorinated organic pesticides (Rao, 2006a; Rao, 2006b; Pandcy et al., 2006; Capkin et al., 2006).

For the nitrogen group the analysis of ammonia nitrogen, nitrite nitrogen and nitrate nitrogen indicated presence of low concentrations of all. Un-ionized ammonia is the most toxic parameter of fish in aquaculture sector and polluted water (Zhao et al., 1997; Harris et al., 1998; El-Shafai et al., 2004). In fresh water, the toxic levels of free ammonia for short-term exposure usually lie

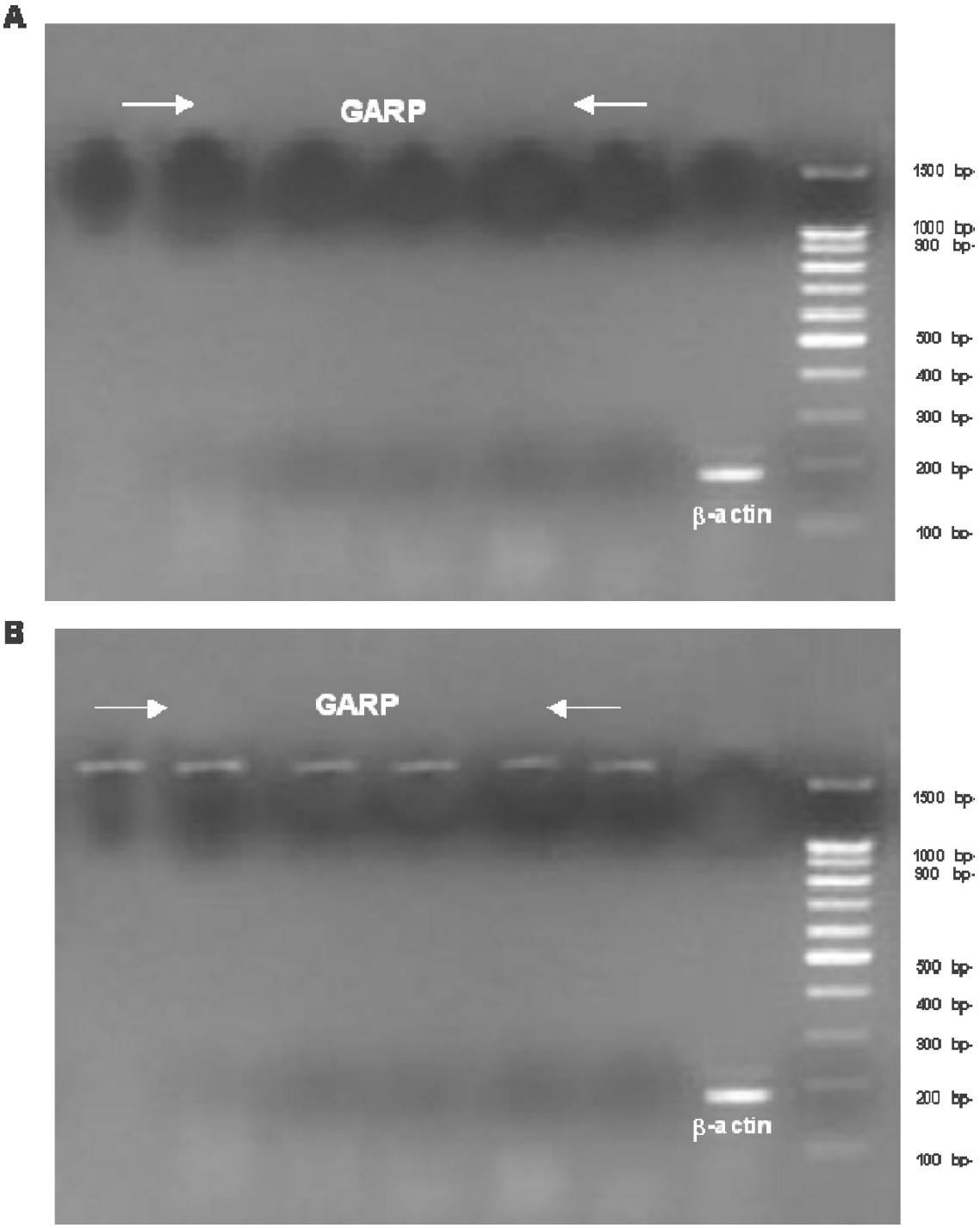

Figure 1. RT-PCR of GARP expressed immune gene of Egyptian sole (*Solea aegyptiaca*) and normalized on the bases of β–actin expression. A) Gel electrophoresis represents PCR products of control fish. B) Gel electrophoresis represents PCR products of polluted fish collected from Lake Qarun.

between 0.6 and 2 mg/l, while others consider the maximum tolerable concentration to be 0.1 mg/l (Pillay, 1992). In this study the ammonia concentrations ranged between 0.12 to 0.52 mg N/l with 0.34 mg N/l an average value. This low concentration is not toxic at all specially with the recorded values of chloride and salinity.

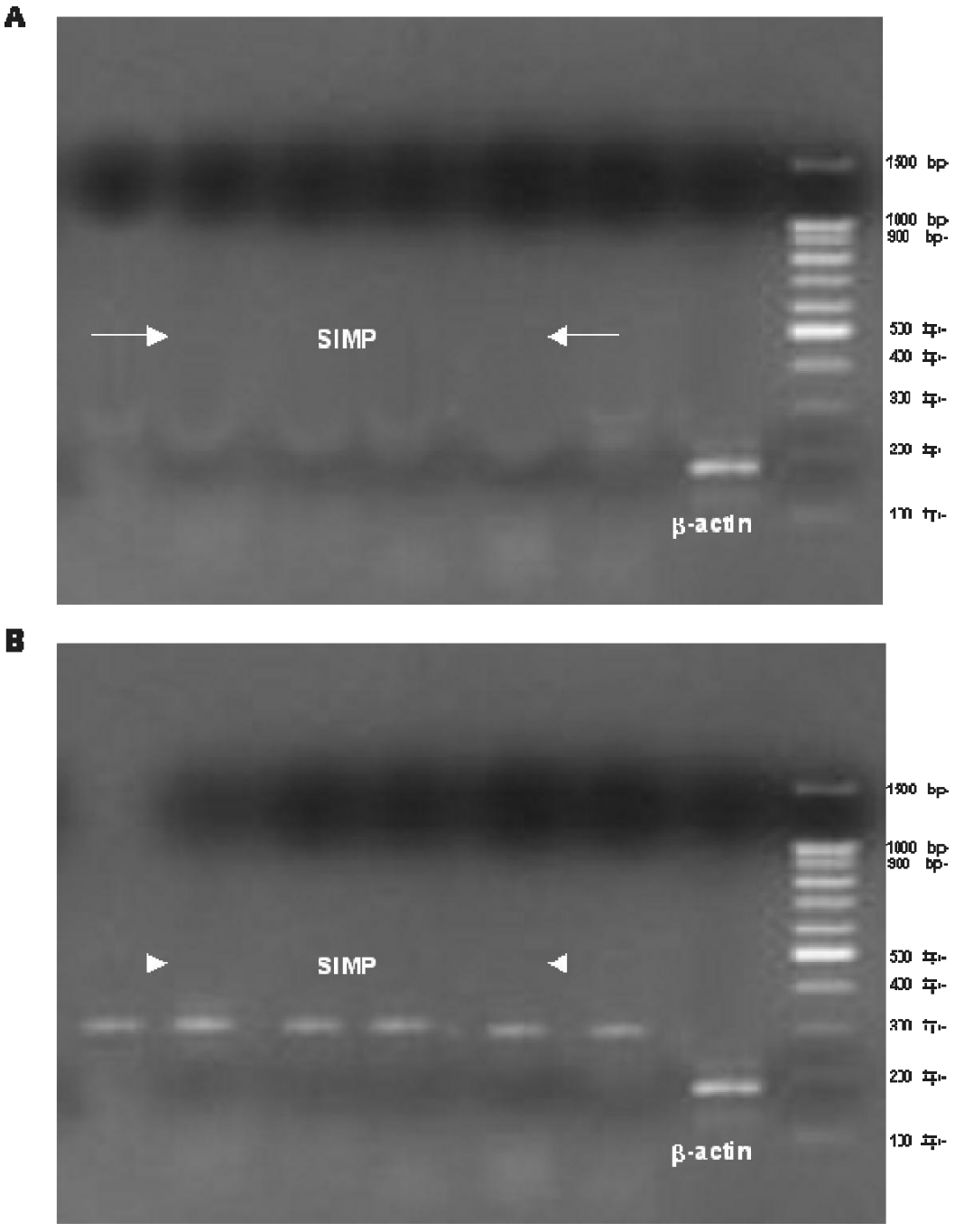

Figure 2. RT-PCR of SIMP expressed immune gene of Egyptian sole (*Solea aegyptiaca*) and normalized on the bases of β–actin expression. A) Gel electrophoresis represents PCR products of control fish. B) Gel electrophoresis represents PCR products of polluted fish collected from Lake Qarun.

The same trend has also been observed for nitrite, which causes brown blood disease in fish and blue babies syndrome in infants. Its toxicity is attributed to oxidation of hemoglobin into non-functional methemoglobin (Colt et al., 1981). In this study, the nitrite concentrations recorded an average value of 0.03 mg N/l and a maximum value of 0.11 mg N/l. The ammonia and nitrite are two of the major pollutants threaten the health of aquatic organisms especially in fresh water. In brackish and marine water the toxic levels of both ammonia and

nitrite is higher and are rarely reached in the environment.

The presence of nitrite and ammonia could be attributed to the presence of sewage bacteria coming with the inlet water. It is very known that the drains, which receive treated and untreated sewage, are the main water feed of Lake Qarun. The low dissolved oxygen and nitrite toxicity increase susceptibility of the fish to bacterial infection (Bunch and Bejerano, 1997). The concentration of total pesticides recorded in the lake (0.62 mg/l) is high enough to cause some alteration in the biochemical and physiological parameters in fish. Sub-lethal concentration (0.017 mg/l) of some organo-phosphorous pesticides causes adverse effects on fish (Rao, 2006a; Rao, 2006b). On the other hand the toxicity of sub-lethal con-centration of some organo-chlorinated pesticides reaches low to 0.002 mg/l. This is according to the results of Pandcy et al. (2006) and Capkin et al. (2006). The results indicated that the 24-h LC50 for endosulfan, organo-chlorinated pesticides is 0.02 mg/l.

The higher TVBC is an indication of microbial load in the Lake. Presence of STC confirms occurrence of faecal pollution (WHO/UNEP, 1995). The inlet point of the drains in the Lake showed considerable number of coliform group and also near the shore of the Lake. This agrees with Sabae and Rabeh (2000) and referred to direct disposal of sewage into these drains or near the shore. SFC confirms the recent discharge of animal or human excrements to the drains or near the shore of Lake. We tentatively suggest that faecal coliforms also remain active in marine water. The duration of this active period in marine water is unknown at present; however, research is underway to elucidate this phenomenon.

To determine the influence of pollution on the immune response, gene expression of some immune genes were analyzed in Egyptian sole (*S. aegyptiaca*) fish. As verified by RT-PCR, GARP and SIMP were highly up-regulated in livers from Egyptian sole (*S. aegyptiaca*) fish. However, GARP and SIMP genes were down-regulated in the livers from control Egyptian sole (*S. aegyptiaca*) fish. This might probably be related to the microbial infection (Chang et al., 2005b). Total viable bacterial count in the present study was fluctuated between around 10^3 and 10^6 CFU/ml. Also presence of pesticides with 0.62 mg/l might be the reason.

It is expected that pollution and microbial infection in vertebrates would evoke an immune response. In the present study, the pollution and microbial contamination of Lake Qarun stimulated the immune response in Egyptian sole (*S. aegyptiaca*) fish. Chang et al. (2005a) found a few protein components of acute-phase response (APR) in grass carp in response to parasite infection as part of the innate immune defense. APR was usually characterized with the change of plasma proteins which are referred to as acute-phase proteins (APPs), and also characterized with the secretion of some other innate defense molecules (Bayne et al., 2001). Bayne et al. (2001) also identified most of these components in the up-regulated libraries from livers of infected rainbow trout (*Oncoryhnchus mykiss*). We can also suggest that the up-regulated expression of GARP and SIMP genes in Egyptian sole (*S. aegyptiaca*) fish as confirmed by RT-PCR may also indicate that APPs are expressed and capable of up-regulation during the pollution and microbial infection in Lake Qarun. In addition, the identification of known or novel genes involved in the pollution provides the foundation for further study on the immunological interaction between the fish host and the microorganism, and it appears possible that construction of subtractive library may be useful for studying immune mechanisms to microbial infection.

Conclusion

The study concludes that the water quality in Lake Qarun is polluted and this pollution enhances up-regulation of some immune genes of Egyptian sole (*S. aegyptiaca*) fish. The application of immune gene expression analysis might be timely, safe and cost effective method to detect the water quality with different sources of pollution.

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