African Journal of Biotechnology Vol. 6 (7), pp. 840-846, 2 April 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Estimation of water pollution by genetic biomarkers in tilapia and catfish species shows species-site interaction

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Accepted 20 February, 2007

This study was aimed at the estimation of water pollution with heavy metals using four biomarkers as well as to study the species-site interaction. Two species of tilapia as well as two catfish species caught from four sites that represent differential environmental stresses were used for this purpose. Water samples and gills were analyzed for heavy metal contents. Three enzyme biomarkers (acid phosphatase (AP), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), metalothionein (MT) gene expression) as well as real time PCR quantification of metalothionein transcripts from liver were used to monitor fish response to water pollutants. Results showed various activities of the four biomarkers at the different studied sites. There were clear interaction between fish species and the level of heavy metals. Real time PCR evaluation of metalothionein gene expression revealed species variations at similar sites. It is concluded that there are various types of interaction of species at different sites. These types of interaction depend on the type of biomarker tested.

Key words: Water pollution, heavy metals, metalothionein, catfish, tilapia, real time, PCR, GOT, GPT, acid phosphatase.

INTRODUCTION

Aquatic animals have often been used in bioassays to monitor water quality (Carins et al., 1975; Brugs et al., 1977). The development of biological monitoring techniques based on fish offers the possibility of checking water pollution with fast responses on low concentrations of direct acting toxicants (Poele and Strick, 1975; Badr and El-Dib, 1978; Ali and El-Shehawi, 2006).

Fish are excellent subjects for the study of various effects of contaminants present in water samples since they can metabolize, concentrate, and store waterborne pollutants. Since fish often respond to toxicants in a similar way to higher vertebrates, they can be used to screen for chemicals that are potentially teratogenic and carcinogenic in humans. The main application for model system using fish is to determine the distribution and effect of chemical contaminants in the aquatic environment (Al-Sabti and Metcalfe, 1995).

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Pollutants act by changing the structural or biological functions of biomolecules (Newman, 1998). Biomarkers for water pollution are early diagonositic tools for biological effect measurement and environmental quality assessment (Cajaraville et al., 2000). They are defined as a change in biological response that differs from molecular to organismal level (Depledge et al., 1995). Tilapia and catfish are among many fish species that are used for this purpose. They represent different sensitivity for environmental pollutants.

The biological effects of water pollutants were measured in Lake Mariout using *Tilapia nilotica* as bioindicator. Samples were examined for the activities of acetylcholinesterase, alkaline phosphatase, and glutathione S-transferase. Levels of cadmium (Cd) and mercury (Hg) were determined in both fish organs and water. Also, the electrophoretic patterns of protein were presented as well as the amino acid composition of fish protein. Water samples of Lake Mariout contained high concentrations of Hg and Cd that were associated with differences in electrophoretic patterns of proteins prepared from Mariout and other locations (El- Demerdash and Elagamy, 1999).

Acid phosphatase (AP), glutamic oxaloacetic (GOT) and glutamic pyruvic (GPT) transaminases are among many enzymes that are commonly used as biomarkers of environmental pollution. Acid phosphatase was used to estimate the effect of heavy metals pollutants as indicated by the analysis of water samples and gills of *Cyprinus carpio* (Ozmen et al., 2006). The enzyme was employed in fish liver to study the effects of the extensive dredging in Goteborg harbor situated at the river Gota alvestuary, Sweden using Eelpout (*Zoarces viviparus*) (Sturve et al., 2005). Liver glutamic oxaloacetic (GOT) and glutamic pyruvic transaminases (GPT) were used to assess the impact of long-term exposure to waterborne cadmium (Cd) on *C. carpio*. Both showed increased activity in response to cadmium (De la Torre et al., 2000). The effect of lead and copper on certain biochemical parameters of the aquatic insect *Sphaerodema urinator* has also been estimated. The results showed an increase in the activity of acid phosphatase. Also the treated insects showed lower activities of GOT and GPT (Bream, 2003). GOT and GPT were employed to estimate the effect of accumulated residues of DDT, DDE, aldrin, dieldrin and deltamethrin. Higher level of GPT and GOT was found in samples with higher accumulation of pesticide residues. This possibly indicates a correlation between exposure of pesticide and increased level of the two enzymes (Saqib et al., 2005).

Change in gene expression is used as biomarker for exposure to pollutants. The expression level of metalothionein gene in fish has been used as a biomarker for water pollution with heavy metals (Evans et al., 2000; Evans et al., 2001, Tom et al., 2004, Sturve et al., 2005). The gene bank has the full cDNA for metalothionein for *Oreochromis aureus* (Accession #: 30144558) and *Oreochromis mossambicus* (OM, Accession #: 30144562). This offers a good opportunity for designing primers for real time PCR quantification of metalothionein expression in response to pollution with heavy metals.

This work was planned to estimate to monitor water pollution employing enzyme biomarker as well as the expression level of fish metalothionein gene. In addition, the study was designed to reveal the interaction between different fish species and the tested locations and compare the differential response of species to pollutants. For this purpose, four sites that display differential environmental stresses were selected and two species of tilapia as well as two species of catfish were utilized. The difference and the relationship between the two types of biomarkers are discussed.

MATERIALS AND METHODS

Fish and water samples

Fish; *Oreochromas niloticas* (ON), *Tilapia zillii* (TZ), *Clarias gariepinus* (CG), and *Clarias lazera* (CL); and water samples were collected from four sites (Ali and El-Shehawi, 2006). These sites represent differential environmental stresses. Site 1 is the River Nile (Shubrakhit). It is considered as the control since it is the common source for irrigation and drinking in Egypt. Site 2 is a closed drainage at Abou Homos. Site 3 is drainage at Kafr Eldawar (Barsiwqe). Site 4 is Lake Mariout.

Preparation of samples for heavy metals concentration

Heavy metal concentrations were estimated in water samples as well as fish gills. These organs were collected and kept frozen until use. Specific weight of fish gills were homogenized and dried. The heavy metals cadmium copper, and lead were determined using atomic absorption Spectrophotometer (Perkin-Elmer, 2380) using standard curve for each metal.

Enzyme activity measurements

Fish were caught from the four specified sites. Fish were dissected alive. Liver was removed rapidly and cleaned of accessory connective and adipose tissues. Tissues were homogenized in 10 volumes (w/v) of ice cold phosphate buffer (0.1 M, pH 8.0) using polytrone homogenizer for 20 s. The homogenates were then centrifuged at 6000 X g for 30 min at 4ºC. The supernatant was used for the measurement of each enzyme activity.

Acid phosphatase activity (AP)

Acid phosphatase activity was determined using Sodium *p*-nitrophenylphosphate as substrate. Buffer/substrate solution (0.5 M citrate buffer, 0.0055 M *p*-nitrophenylphosphate, pH 4.8) to 10 mL of homogenate was added. The reaction mixture was incubated for 30 min at 37ºC. 4 mL of 0.1 M NaOH was then added to stop the enzymatic reaction. The absorbance was measured at 400 nm. The activity of acid phosphatase was expressed as units per mg protein.

Transaminase activities (GOT and GPT)

Activities of GOT and GPT were assayed using a commercially available kit according to the producer instructions. Colorometric determination of GOT and GPT activity was carried out according to the following reactions:

1. Glutamate oxaloacetate transaminase (GOT)

Aspartate + α-ketoglutarate → oxaloacetate + glutamate

2. Glutamate pyruvate transaminase (GPT)

Alanine + α-ketoglutarate → Pyruvate + glutamate

The oxaloacetate or pyruvate formed was measured in its derivatives from 2,4-dinitrophenylhydrazone at 505 nm. Phosphate buffer/substrate (0.5 ml), pH 7.5 was incubated for one hour for GOT or 30 min for GPT. A 0.5 ml of color reagent, 2,4-dinitrophenyldrazine, was then added. The incubation was continued for 20 min at room temperature. Finally, 5 ml of 0.4 N sodium hydroxide was added, mixed well, and kept for 5 min at room temperature. The absorbance was measured at 505 nm. Activity was expressed as units/mg protein.

Sequence alignment of metalothionein gene

Blast software (Altschul et al., 1990) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) was used to align the metalothionein gene sequence from *O. aureus* (Acces-

Table 1. Average of concentration of heavy metals in water samples of the four sites.

Site	Cadmium (ppm ± SE)	Lead (ppm ± SE)	Copper (ppm ± SE)
1	14.2 ± 0.83[b]	20.5 ± 1.3[b]	2.4 ± 0.8[d]
2	8.11± 0.25[c]	7.88 ± 0.8[c]	7.33 ± 0.45[b]
3	30.4 ± 0.82[a]	6.43 ± 0.12[d]	4.42 ± 032[c]
4	7.41 ± 0.23[d]	36.14 ± 0.1[a]	27.12 ± 1.3[a]

LSD Cadmium = 0.46, LSD Lead = 0.19 and LSD Copper = 0.24.
Small letters indicate significance in columns ($p < 0.05$).

sion #: 30144558) and *O. mossambicus* (Accession #: 30144562). Both sequences have three exons 25, 66, and 92 bp, respectively.

Primer design

Forward and reverse primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) using the identical sequence in both accessions. The forward primer anneals at 1006 in the 5' region of exon 2 (5' CTGCAAGAGCTGCAAGAAGA 3') and the reverse primer anneal at 1342 in the 5' region of exon 3 (5' TGTCGCATGTCTTTCCTTTG 3'). This pair of primers will amplify 95 bp fragment. The consensus 18S rRNA primer pair 18S1 (forward, 5'-TACCACATCC AAAGAAGGCA C-3') and 18S2 (reverse, 5'-TCGATCCCGA GATCCAACTA C-3') (Tom et al., 2004) was used to amplify 245 bps fragment of the 18S rRNA as standard for the real time PCR reaction.

RNA isolation

Total RNA was isolated from liver of fish species caught from the four sites under investigation using TriReagent solution (MRC, USA) according to manufacturer instructions. RNA was fractionated on 1% agarose gel to check its quality and its concentrations were measured by spectrophotometry.

Real time PCR

Liver metalothionein transcript was determined using one step QuantiTect Cyber Green RT-PCR kit (Qiagen) according to the manufacturer instructions and the iCycler iQ Real Time PCR (Biorad, USA). This included the reverse transcription for 30 min at 50ºC, initial activation step at 95ºC for 15 min, 45 cycles of denaturation for 15 s at 95ºC, annealing for 30 s at 55ºC, and extension for 30 s at 72ºC. Ten nanogram of total RNA (1×10^{-3} diluted) were included as template for metalothionein transcript. Serial dilutions (1×10^{-2}, 1×10^{-4}, 1×10^{-6}, 1×10^{-8}, and 1×10^{-10}) of total RNA were prepared for evaluation of 18S transcript for the standard curve.

Statistical analysis

Data were analyzed using two-way ANOVA using site and species as the main two factors. This was followed by comparisons of means by LSD at 95% significance level (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Analysis of water samples

Analysis of heavy metals in water samples of the four sites under study showed that there is a significant difference of each heavy metal among the four sites including the control. The results revealed that there were differences in various heavy metals in the same site. Site 1 showed the highest concentration of lead (20.5 ± 1.3) and the lowest of copper (2.4 ± 0.8). Site 2 gave close concentrations of the three heavy metals analyzed (8.11 ± 0.25, 7.88 ± 0.8, 7.33 ± 0.45 for cadmium, lead, and copper, respectively). Site 3 showed the highest concentration of cadmium (30.4 ± 0.82) while it showed the lowest concentration of lead (6.43 ± 0.12). Site 4 indicated the highest concentration of lead (36.14 ± 0.1) and copper (27.12 ± 1.3) and the lowest of cadmium (7.41 ± 0.23). Table 1 summarizes the data of water analysis of the three heavy metals collected from the four sites. The data for site 4 can be explained because this site (Lake Mariout) is highly contaminated with some heavy metals (El-Demerdash and ELagamy, 1999), but these concentrations are subject to changes due to new deposition of pollutants explaining the need for regular monitoring of water resources.

Cadmium concentration in gills of the four fish species showed significant differences among all species. *C. lazera* showed the highest average (3.4), whereas *T. zillii* showed lowest the average (2.1) (Table 2). In addition, there are significant differences among the average concentration of the different sites. Furthermore, there is an interesting pattern of interaction between certain species and specific site. For example, *C. gariepinus* showed the highest concentration of cadmium in gills in site 3, while *O. niloticas* showed the lowest interaction in site 4. Table 2 summarizes the cadmium averages in gills. This data indicate that different species have various capabilities to filtrate and store water contaminants independent of their level in water. This phenomena was previously reported by De la Torre et al. (2000).

Table 3 summarizes the average values of lead concentration in gills of the four fish species caught from different sites. *T. zillii* showed the highest mean (4.5)

Table 2. Concentration of cadmium in gills of the four fish species collected from the four studied sites).

Site	ON ppm ± SE	TZppm ± SE	CG ppm ± SE	CL ppm ± SE	Mean, sites
1	2.3 ± 0.06	2.4 ± 0.12	2.3 ± 0.07	4.2 ± 0.12	^{B}2.8
2	1.4 ± 0.12	1.7 ± 0.05	3.2 ± 0.02	3.1 ± 0.07	^{C}2.4
3	4.4 ± 0.14	3.1 ± 0.01	5.1 ± 0.06	4.11 ± 0.01	^{A}4.18
4	1.2 ± 0.01	1.1 ± 0..02	1.2 ± 0.05	2.1 ± 0.06	^{D}1.4
Mean, Species	^{c}2.3	^{d}2.1	^{b}2.95	^{a}3.4	

LSD sites = 0.14, LSD species = 0.14, LSD interaction = 0.28
Small letters indicate significance in rows ($p < 0.05$).
Capital letters indicate significance in columns ($p < 0.05$).
ON: *Oreochromas niloticas*; TZ: *Tilapia zilli*; CG: *Clarias gariepinus*; CL: *Clarias lazera*.

Table 3. Concentration of lead in gills of the four fish species collected from the four studied sites.

Site	ON ppm ± SE	TZ ppm ± SE	CG ppm ± SE	CL ppm ± SE	Mean, sites
1	4.0 ± 0.0	5 ± 0.58	3.1 ± 0.06	3.12 ± 0.01	^{A}3.8
2	2.2 ± 0.12	3 ± 0.59	2.4 ± 0.07	1.1 ± 0.05	^{B}2.2
3	2.4 ± 0.13	3 ± 0.56	1.3 ± 0.05	1.2 ± 0.06	^{B}2.0
4	4.7 ± 0.0	7 ± 0.06	4.1 ± 0.04	4.2 ± 0.13	^{A}5.0
Mean, Species	^{ab}3.3	^{a}4.5	^{b}2.7	^{b}2.4	

LSD sites = 0.6, LSD species = 0.6, LSD interaction = 1.2
Small letters indicate significance in raws ($p < 0.05$).
Capital letters indicate significance in columns ($p < 0.05$).

Table 4. Concentration of copper in gills of the four fish species collected from the four studied sites.

Site	ON ppm ± SE	TZ ppm ± SE	CG ppm ± SE	CL ppm ± SE	Mean, sites
1	2.1 ± 0.05	2.4 ± 0.12	2.2 ± 0.12	1.2 ± 0.05	^{D}2.0
2	6.2 ± 0.12	1.8 ± 0.14	4.2 ± 0.12	2.2 ± 0.04	^{B}3.6
3	4.4 ± 0.13	3.1 ± 0.01	2.4 ± 0.06	1.2 ± 0.05	^{C}2.8
4	7.6 ± 0.12	1.1 ± 0.0	8.1 ± 0.05	6.4 ± 0.07	^{A}5.8
Mean, Species	^{a}5.1	^{d}2.1	^{b}4.2	^{c}2.8	

LSD sites = 0.15, LSD species = 0.15, LSD interaction = 0.3
Small letters indicate significance in rows ($p < 0.05$).
Capital letters indicate significance in columns ($p < 0.05$).

which was significantly different from the other three species. This was very clear in site 4 (7 ± 0.06). There were also significant differences among the mean of sites. Site 4 gave the highest average (5.0). There was also an interaction between sites and species. This interaction was indicated in that *C. lazera* had the lowest interaction in site 2, whereas *T. zillii* showed the highest interaction in site 4.

Concentration of copper in gills is presented in Table 4. Results revealed that there were significant differences among fish means as well as among site means. *O. niloticas* gave the highest mean (5.1), whereas *T. zillii* gave the lowest one (2.1). Regarding sites, site 4 had the highest mean, while site 1 had the lowest one. In addition, an interaction between fish species and the level of pollutants in different sites was also observed. *T. zillii* showed the lowest value for interaction in site 4, whereas *O. niloticas* showed the highest value in site 4.

The activity of the acid phosphatase (AP) of the four fish species caught from the four studied sites was measured. Significant differences among fish averages as well as site averages were observed. The enzyme activity was highest in site 4 and was lowest in site 1, whereas *T. zillii* gave the highest average, while *C. gariepinus* gave the lowest average. The species-site interaction was maximum in *O. niloticas* in site 4 and was minimum in *C. gariepinus* in site 1.

Glutamic Oxaloacetic Transaminase (GOT) was measured in blood samples drawn from fish caught from the specified sites. The results are summarized in Table 6. Different fish species as well as different sites gave significantly different means. *C. lazera* gave the highest

Table 5. Liver acid phosphatase activity (u/mg protein) of the four fish species collected from the studied sites.

Site	ON ppm ± SE	TZ ppm ± SE	CG ppm ± SE	CL ppm ± SE	Mean,sites
1	2.66 ± 0.04	4.1 ± 0.16	2.2 ± 0.24	5.1 ± 0.18	^{D}3.5
2	3.66 ± 0.1	7.8 ± 0.23	3.2 ± 0.4	6.1 ± 0.2	^{C}5.2
3	6.5 ± 0.14	8.1 ± 0.3	2.4 ± 0.09	6.1 ± 0.31	^{B}5.8
4	8.77 ± 0.08	11.1 ± 0.12	3.1 ± 0.05	8.0 ± 0.24	^{A}7.8
Mean, Species	^{c}5.4	^{a}7.8	^{d}2.7	^{b}6.4	

LSD sites = 0.000059, LSD species = 0.000059, LSD interaction = 0.001
Small letters indicate significance in rows ($p < 0.05$).
Capital letters indicate significance in columns ($p < 0.05$).

Table 6. Activity of blood GOT (u/mg protein) of the four fish species caught from the studied sites.

Site	ON ppm ± SE	TZ ppm ± SE	CGppm ± SE	CL ppm ± SE	Mean, sites
1	32.2 ± 0.8	40.2 ± 0.4	32.4 ± 1.27	60.2 ± 0.8	^{C}41.2
2	40.4 ± 1.3	60.5 ± 2.1	42.2 ± 2.34	84.3 ± 1.2	^{B}56.9
3	22.2 ± 0.44	43.5 ± 0.7	38.2 ± 1.1	36.5 ± 0.9	^{D}35.1
4	84.4 ± 3.6	96.3 ± 1.31	54.2 ± 0.9	66.2 ± 0.7	^{A}75.3
Mean, Species	^{c}44.8	^{b}60.1	^{d}41.8	^{a}61.8	

LSD sites = 0.22, LSD species = 0.22, LSD interaction = 0.44
Small letters indicate significance in rows ($p < 0.05$).
Capital letters indicate significance in columns ($p < 0.05$).

Table 7. Average values of the activity of blood GPT (u/mg protein)of the fish species caught from the studied sites.

Site	ON ppm ± SE	TZ ppm ±SE	CG ppm ± SE	CL ppm ± SE	Mean, sites
1	14.2 ± 1.1	30.3 ± 1.9	12.2 ± 0.8	28.4 ± 2.4	^{D}21.3
2	17.3 ± 0.78	44.2 ± 1.12	18.2 ± 0.7	33.2 ± 1.5	^{C}28.2
3	32.2 ± 2.0	48.3 ± 0.87	16.2 ± 2.3	24.2 ± 0.65	^{B}30.2
4	34.5 ± 0.92	12.4 ± 1.6	36.4 ± 1.3	48.2 ± 0.4	^{A}32.9
Mean,Species	^{c}24.55	^{a}33.8	^{d}20.8	^{b}33.5	

LSD A = 0.25, LSD B = 0.25, LSD AB = 0.5
Small letters indicate significance in raws ($p < 0.05$).
Capital letters indicate significance in columns ($p < 0.05$).

mean (61.8), while *C. gariepinus* gave the lowest (41.8) and site 4 showed the highest average (75.3), whereas site 3 showed the lowest average (35.1). *T. zillii* from site 4 gave the highest interaction (96.3 ± 1.31) compared to *O. niloticas* from site 3 that gave the lowest interaction (22.2 ± 44).

The activity of GPT was estimated in blood samples from fish caught from the four sites. Table 7 shows a summary of the obtained results. *T. zillii* gave the highest average (33.8) and *C. gariepinus* showed the lowest (20.8), while site 4 gave the highest average (32.9) and site 1 showed the lowest average (21.3). *T. zillii* caught from site 3 gave the highest interaction (48.3 ± 0.87) compared to *C. gariepinus* caught from site 1 that gave the lowest interaction (16.2 ±2.3) (Table 7).

Clarias species are known to be more tolerant to pollutants. It is observed that they do store significant higher levels of cadmium in their gills (Table 2). On the other hand, this is not the case in lead and copper since Tilapia species showed significant higher concentrations of these two metals in their gills (Table 3 and 4). Moreover, tilapia exibited higher levels of acid phosphatase, especially *T. zillii* (Table 5), whereas *C. lazera* showed more GOT activity. It seems that there are site-related factors probably including other contaminants that are involved in the activities of these biomarkers. Therfore, *Clarias* species tolerate higher levels of pollutants that is indicated by storing high levels of certain metals, such as cadmium in their gills and in the same time show lower enzyme activity, such as acid phosphatase, yet this is not

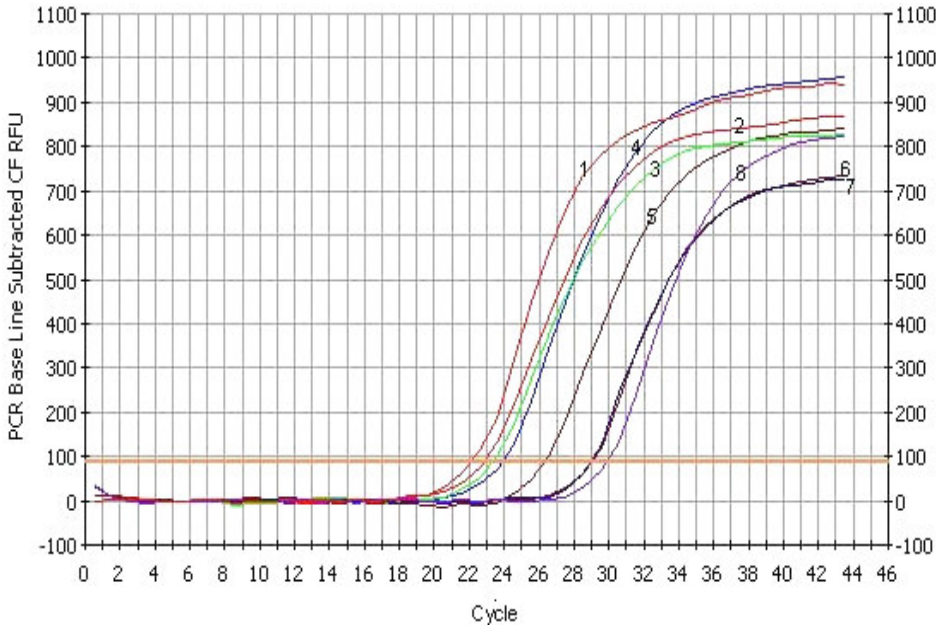

Figure 1. Real time PCR quantitaion of liver metalothionein transcript from *Oreochromas niloticas* and *Tilapia zilli* caught from the four investigated sites. Curves 1 - 4 represent *O. niloticas* at sites 1 - 4 and curves 5 - 8 represent *T. zilli* at sites 1 - 4 respectively.

the case in all metals tested. Differential responses of various biomarkers to pollutants have been reported. GOT and GPT showed increased activity in response to cadmium (De la Torre et al., 2000) when employed to assess the impact of long-term exposure to waterborne cadmium in *C. carpio*. An increase in the activity of acid phosphatase and a decreased activities of GOT and GPT were found in response to lead and copper in the aquatic insect *S. urinator* (Bream, 2003).

Metalothionein gene expression

Metalothionein is a small protein of 60 amino acid residues in fish. The sequence of *O. aureus* (Accession #: 30144558) and *O. mossambicus* (Accession #: 30144562) are the only complete cDNA deposited in the database from fish. Sequence alignment of the two metalothionein gene accessions showed 98% identity at the nucleotide sequence level and 100% at the protein sequence level. The gene has three exons of 25, 66, and 92 bp separated by two introns of 93 and 242 bp respectively.

A pair of primer was designed to amplify the 3' end of exon2 and the 5' end of exon3 as a 92 bp fragment from metalothionein transcript in the four fish species. No amplification products were detected in both *Clarias* species, but they were productive with *O. niloticas* and *T. zilli*. Failing to give products with *Clarias* species could be explained by difference in the sequence of primer annealing and/or different gene organization; different exon/introns organization and number. There are no sequences of metalothionein for *Clarias* species deposited in the database to use for comparison.

Metalothionein transcript evaluation in tilapia species showed that difference between *O. niloticas* and *T. zillii* because the transcript was detected at lower threshold cycles (Ct) in *O. niloticas* at all sites compared to *T. zillii*. The Ct cycles were 22.7, 23.3, 23.8, and 22 at sites 1 - 4 respectively for *O. niloticas*, whereas Ct cycles were 28.9, 28.9, 28.8, and 26.2 at sites 1 - 4 respectively for *T. zillii* (Figure 1). These results revealed that the gene was expressed at higher level in *O. niloticas* than *T. zillii* in response to water pollutants.

The expression data was related to the concentration of heavy metals in water and gills as well as activity of enzyme biomarkers. Site 4 showed high concentration of lead and copper. This was indicated by lower Ct cycles in both fish species; high expression of the gene. Sites 2 and 3 showed close concentrations of heavy metals. This also was related to their Ct cycles for metalothionein expression; similar levels of gene expression in each species.

Conclusion

The results of this study conclude that there were clear interaction between fish species and the level of heavy metals. In addition, real time PCR evaluation of metalothi-

onein gene expression could be employed to estimate heavy metal pollution in water since it showed compatible results with other biomarkers. Moreover, results of this study revealed that there is an independent interaction between each fish species with the water environment. Therefore, using more than one fish species is recommended in the monitoring of water pollution with biomarkers.

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