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ORIGINAL ARTICLE

Potential of the hepatic transcriptome expression profile of the striped seabream (*Lithognathus mormyrus*) as an environmental biomarker

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Abstract

The potential of the hepatic transcriptome expression profile evaluated in a sentinel feral fish to serve as an environmental biomarker was examined. Expression profiles of *Lithognathus mormyrus* individuals were exhibited using cDNA microarray and were related to the set of exposure conditions at their sites and dates of collection. Expression profiles of individual fish were reasonably clustered according to the fish samples. In addition, several sample-specific gene clusters were determined, designated sample gene signatures. The selection procedure for future optimal reference RNA is discussed. The relationship between transcriptome expression and fish samples indicated a potential for using the former as an environmental biomarker.

Keywords: *Aquatic organisms; environmental pollution/ecotoxicology; gene expression; biomonitoring; fish; cDNA microarray*

Introduction

Anthropogenic impact on natural biota is a major environmental threat and its monitoring is essential for designing and adjusting environmental policy. Sentinel organisms inhabiting an examined habitat are exposed to, and affected by, their surroundings. Consequently, pollution-affected biological parameters measured in suitable organs of a sentinel species have been widely suggested as biomarkers of pollution effects (Moore et al. 2004). The subject biomarker in the present study is the transcriptome expression profile evaluated in a sentinel fish. It is a complex parameter composed of the list of relative expression levels of specific genes in an individual specimen or in a defined group of individuals, in comparison to the corresponding levels in a reference transcriptome. The term, expression profile, as used in this study is exactly defined in the Materials and methods section below. Due to its comprehensiveness, this biomarker was postulated to respond sensitively to a wide range of environmental threats.

The potential of evaluating coordinated expression of genes for the detection of the biological effects of environmental pollutants has been discussed in recent literature applying microarray platforms as a detection tool (Ankley et al. 2006, Carvan et al. 2008). Comparability over a broad time scale and wide geographical range is considered an essential requirement for an environmental biomarker and can be achieved by utilizing a common reference RNA. The best theoretical reference is a mRNA mixture taken from individuals that have never been exposed to pollution, more tightly relating gene differential expression in the sampled fish to pollution effect.

A feral fish, the striped seabream, *Lithognathus mormyrus* was chosen as a sentinel species of Mediterranean coastal habitats (Yudkovski et al. 2008). Itisa demersal fish,inhabiting the Israeli coast of the Mediterranean in both polluted and pristine sandy habitats. It interacts with the bottom through its bottom-feeding habits and burial behaviour and its gut usually contains sediment engulfed with ingested food (Froglia 1977, Kallianiotis et al. 2005). It is a protandrous

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hermaphrodite whose immature gonad is an ovotestis, either developing into a testis or an ovary. However, the developed gonad always includes residual tissue of the opposite sex. *L. mormyrus* reaches maturity during its third year (Besseau & Brusle-Sicard 1995) and it exhibits no external sex-distinguishing characters. Hence, it is not possible to determine sex outside the reproduction season. The sex reversal from male to female occurs in a wide size range. Hence, it is difficult to determine the sex of an individual within this range, from its size (Kallianiotis et al. 2005, Bauchot & Hureau 1986, Kraljevic et al. 1995). The reproduction season extends from June to August–September in the Northern hemisphere.

As in other vertebrates, fish liver is commonly involved in xenobiotic detoxification. Hence, studies using *L. mormyrus* as a sentinel fish have adopted quantitative reverse transcription–real-time polymerase chain reaction (qRT-PCR) for evaluation of the hepatic expression of the biomarker genes. These genes included metallothionein, cytochrome P4501A, vitellogenin and zona radiata protein (ZRP), modified to include appropriate specific transcript standards compatible with the target fish (Yudkovski et al. 2008, Funkenstein et al. 2004). In addition, a cDNA microarray was constructed of transcripts cloned from multipollutant-affected *L. mormyrus* livers (Auslander et al. 2008).

Microarray platforms have been used to study the effects of pollutants on multigene expression in fish, mainly through controlled laboratory exposure to pollutants (Carvan et al. 2008). In addition, one field study compared transcriptome expression profiles in different populations of the sentinel European flounder (*Platichthys flesus*) exposed to assumedly different sets of environmental exposure conditions (Falciani et al.2008). The term set of environmental exposure conditions, as used in this study applies to the comprehensive, not necessarily completely defined set, of environmental-conditions prevailing in the sampled site at the date of sampling.

Ideally, the fulfilment of three working hypotheses would make the present spotted hepatic cDNA assemblage of *L. mormyrus* an efficient tool to test the relationships between expression profiles of individual fish and sets of environmental conditions. These hypotheses are: (1) mutual exposure history to pollutants of all individual fish composing a sample, due to their permanent residence in the examined habitat; these fish are assumed to be characterized by similar phenotype and genotype; (2) bias of the hepatic transcript assemblage spotted on the microarray toward pollution-affected genes; (3) different pollution status of the two sampled sites, one being exposed to more severe pollution pressure than the other.

In the context of the present sentinel and the sampled habitats, only indirect and circumstantial evidence exists to validate the depicted hypotheses. Hence, permanent residence in the examined habitat was postulated based on the *L. mormyrus* demersal biology. Similar phenotypes were judged by similar size and reproductive activity, and no genetic background data were available. Bias of the utilized cDNA assemblage toward pollution-affected genes was deduced from the methodology of its construction (Auslander et al. 2008). Evidence indicating different pollution status of the two sampled sites is presented and discussed in the Materials and methods section.

Therefore, a top-down analytical approach was implemented. It started from relating the characteristics of the gene expression profiles of individual fish to the fish samples exposed to the poorly defined site- and time-related sets of environmental exposure conditions. Later, the compatibility of the working hypotheses with the revealed relationships between fish samples and individual gene expression profiles was explored. Valid hypotheses are expected to lead to more uniform within-samples expression profile characteristics than among samples, affected mainly by the mutual exposure of each sample to a specific set of environment exposure conditions. This study and similar future studies, using other species in a variety of habitats, is the first step towards establishing the examined species as an environmental sentinel.

Materials and methods

Fish sampling, habitat characterization and organ processing

Fish were caught alive by gillnetting in February–March 2007 and 2008 at two sites on the Mediterranean coast of Israel, Haifa (35.00°E 32.83°N) and Dor (34.91°E 32.62°N). The sampling plan was aimed at examining changes in the hepatic expression profiles at different dates and sites of collection, assuming that both parameters may affect the pollution pressure. Winter sampling was conducted to avoid individuals during their reproduction season. The Haifa site samples of 2007 and 2008 were designated H7 and H8, respectively, and the corresponding Dor samples were designated D7 and D8.

The Haifa site is located in the southern part of Haifa Bay, just outside the Haifa harbour, near the Haifa urban and industrial region, and in close proximity to the polluted Kishon estuary. The Dor site is located 25 km south of the Haifa site, remote from urban and industrial regions, bordering a marine nature reserve. This circumstantial evidence for different anthropogenic impact at the two sites was further indicated by the levels of mercury and cadmium in the sediment of both sites. The levels of mercury and cadmium at Dor were estimated from

the corresponding levels at the Taninim site, located 9 km south of Dor, at the same water depth. Like Dor, it is remote from urban and industrial regions and the closest available source of pollution data, postulated to represent fairly the Dor site. The 2005 heavy metal evaluation in the sediment of the Haifa and Taninim (34.89°E 32.54°N) sites (Herut et al. 2006) revealed 0.270–0.919 and 0.061–0.270 $\mu g\,g^{-1}$ dry weight mercury levels and 0.08–1.54 and 0.04–0.07 $\mu g\,g^{-1}$ dry weight cadmium levels in the sediments of the two sites, respectively.

It addition, evaluation of the hepatic cytochrome P4501A transcript levels in *L. mormyrus* sampled at the Haifa and Dor sites in 2004–5 revealed sevenfold higher levels in the former site (Yudkovski et al. 2008). Hence, the two sites can be reasonably postulated to be under different pollution impacts, Dor being the cleaner of the two.

Fish were sacrificed by decapitation 3 h after sampling, followed by immediately dissecting their liver and gonads. The livers were divided into smaller aliquots, snap-frozen in liquid nitrogen, and stored at -80°C for several days, prior to RNA extraction. Gonads were fixed in 4% formaldehyde and their reproductive activity was determined by microscopic observation of fixed gonads smeared between two slides, measuring the average ova diameter of 20 oocytes. Ten reproductively arrested individuals were selected for each sample, totalling 40 fish. The biological characteristics of the samples are presented in Table 1 revealing statistically significant uniform fish weight and length among the four ten-fish samples (one-way ANOVA, df=3, F(weight)=0.357, F(length)=0.195, p <0.05). No reproductive activity was observed, judged by oocyte diameter <100 μm, previously correlated to absence of vitellogenin in the blood (Funkenstein et al. 2004). Due to the *L. mormyrus* biological characteristics it was impossible to determine the sex of individuals sampled during this study (see Introduction above).

Total hepatic RNA was extracted from the livers using the EZ II kit (Biological Industries, BeitHaemek, Israel) according to the manufacturer's instructions. mRNA was purified from the total RNA using the PolyATtract kit (Promega, Madison, WI, USA). The quality of all produced RNA was evaluated by electrophoresis on a 1% agarose-formaldehyde gel and their concentrations were evaluated by Nanodrop spectrophotometry (Nanodrop Technologies, Wilmington, DE, USA).

Table 1. Characteristics of sampled fish.

Sample site and date	Fish weight[a] (g) ± SD	Fish length[b] (mm) ± SD	Oocyte diameter[c] (μm) ± SD
Haifa 2007	46.3 ± 6.7	155 ± 7	68 ± 42
Haifa 2008	45.8 ± 7.5	155 ± 8	40 ± 10
Dor 2007	44.1 ± 6.4	153 ± 8	63 ± 35
Dor 2008	44.6 ± 8.4	152 ± 9	49 ± 31

[a]Fish weight and length was not significantly different among samples (one-way ANOVA, df=3, F(weight)=0.357, F(length)=0.195, p >0.05); [b]fish length indicates an age of one year (Kallianiotis et al. 2005); [c]no-vitellogenin transcript was found in livers of fish with oocyte diameter <100 μm, indicating reproductive arrest (Funkenstein et al. 2004).

Microarray hybridization and analysis

The general construction procedure of the utilized microarray followed Auslander et al. (2008), with modification, to reduce the number of redundant sequences. The final gene assemblage contained 1152 unique cDNA fragments, printed in two separate duplicate sets. Each spot duplicate, which represented a specific DNA fragment was designated a clone. The microarray platform characteristics were deposited in the Gene Expression Omnibus (GEO) database of the American National Center for Biotechnology Information (NCBI) (GEO platform accession no. GPL8143).

The hybridization and imaging procedures followed Yudkovski et al. (2007). Briefly, the indirect labelling was accomplished by replacing part of the RNA uracils by amino allyl-dUTP through reverse transcription of the target mRNA, followed by chemical coupling of Cy3 and Cy5 mono-reactive succinimidyl ester-derivatized fluorescent dyes to the reactive allyl groups. Reference RNA solution was composed of an equalized RNA mix of all fish sampled at the Dor site in 2007, the first sampling year. Any hepatic RNA of the sentinel fish can serve as a technical relative reference solution for creating comparable gene expression profiles. However, the choice of a reference related to a specific set of exposure conditions was postulated to have potential benefit. The reference RNA issue is discussed below in relation to the results of the study. Labelled cDNA from each individual fish was hybridized to two dye swapped arrays versus the common reference cDNA. GenePix Pro 6.1 software (MDS Analytical Technologies, Concord, Canada) was used for slide imaging. The term expression ratio, throughout this study is determined by M=$\log_2$ (normalized Cy3/normalized Cy5) (Yudkovski et al. 2007). M values were calculated for the expression level of each clone in individual fish, compared with its level in the reference cDNA mixture. In addition, weighted sample M was calculated for each clone. $M \neq 0$ indicated differential expression in relation to the reference RNA. The transcript expression profile, referred to the list of M values characterizing an individual fish or a fish sample. Linear Models for MicroArray data (LIMMA) software (Smyth 2004, 2005 and the LIMMA user guide) was used for the calculation of M for each clone in individual fish and weighted M values in fish samples. Extreme differentially expressed spots were manually flagged on the Cy3–Cy5 scatter plot created by the GenePix Pro 6.1, and did not participate in the dual label normalization performed later by LIMMA. Cy5 and Cy3 intensities within each slide were equalized

using the global LOWESS normalization procedure. Only spots with fluorescence intensity >800 (out of a maximum of 65536) in at least one of the dual-labelled dyes and which agreed with the GenePix Pro 6.1 eligibility criteria were included in the LIMMA performed analysis. The 800 unit threshold was arbitrarily defined to avoid weak and susceptible spots. No background subtraction was applied by LIMMA as the area under each spot was protected from non-specific fluorescent signal, revealing lower signal than the background when not specifically hybridized. LIMMA was used also to test statistically the hepatic differentially expressed genes in each fish sample using the linear model statistical testing. Multiple testing correction according to Benjamini and Hochberg (1995) was applied through LIMMA, and *p*-values are actually adjusted*p*-values. A weighted *M* value was calculated from the duplicate spots of each clone using the 'interduplicate correlation method' at the linear modelling step of LIMMA (for mathematical details see Smyth 2004, 2005). Clones were defined in this study to be differentially expressed if elucidating statistical difference from M=0 (p<0.01) and if revealing $M > |0.5|$. The two stringent thresholds were aimed at minimizing false-positive *M* values resulting from suspected weak differential expression. EXPANDER 5.1 (Sharan et al. 2003 and EXPANDER 5.1 user guide) was used for clustering the expression profiles of individual fish and of the four samples, alternatively applying complete, average and single linkage procedures to create hierarchical clusters. Pearson's correlation was used to calculate similarity and the dissimilarity index present in the graphical presentations below is calculated as (1– Pearson's correlation coefficient)/2. Pearson's correlation coefficient spans between 1 and–1, hence the dissimilarity index obtains values of 0–1.

Clones that demonstrated similar differential expression directions across the various samples were assigned to one clone cluster. The elucidated clones were not always differentially expressed in all samples in view of our selection criteria and M=0 was assigned to each of these non-differential expression results.

Microarray-based analytical procedure and definitions of biomarker indices

The following analytical steps were conducted for the formation of biomarker indices based on multitranscript expression profiles: (1) expression profiling of each of the clones across the fish samples and selection of differentially expressed clones; (2) hierarchical clustering of expression profiles of individual fish; (3) hierarchical clustering of sample weighted expression profiles; and (4) clustering of clone expression profiles across samples.

Two parameters were used as biomarker indices: (1) the number of differentially expressed clones in each fish sample and their average positive and negative *M* values; and (2) sample-specific clone clusters, termed expression profile signatures. These signatures were characterized by their participating clones and by the average *M* values of all up- or downregulated clones. Available annotated gene functions were also part of the signature characterization.

Absolute and relative qRT-PCR

Metallothionein, cytochrome P4501A, ZRP and β-actin transcript levels were evaluated by absolute qRT-PCR (StepOne plus; Applied Biosystems, Foster City, CA, USA) according to Funkenstein et al. (2004) and Yudkovski et al. (2008).

Relative qRT-PCR assays were also performed according to Yudkovski et al. (2007) using the primer pairs described in Table 2. Their purpose was to validate the microarray results. Briefly, the difference between the real-time PCR cycles of selected transcripts in selected individuals and the parallel expression level of the reference RNA solution (ΔCP), were compared with the corresponding *M* values taken from the microarray results. Both *M*-values and ΔCPs represent $\log_2$ expression ratio between compared RNA populations. Although evaluated by different methods, they can be qualitatively compared, demonstrating expression trends.

Total RNA and β-actin served as alternative normalizing agents in both the relative and the absolute qRT-PCR.

Results

Analysis of hybridized slides

Differential expression of each clone in the four fish samples was analysed by LIMMA. The resulting dataset has been deposited in the NCBI Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO Series accession number GSE2262.

It should be emphasized that all eligible clones were included in this analysis, even if available only in part of the 40 analysed fish, and 130 clones were differentially expressed in at least one sample. All the clones that appeared as differentially expressed in more than one sample exhibited a uniform trend of up- or downregulation across samples. Clones with different sequences that share annotations demonstrated agreeable trend of expression direction when comparing their differential expression in the same sample, excluding apolipoprotein 14kDa (acc. no. DQ849911) which revealed an opposite

Table 2. Quantitative reverse transcription–real-time polymerase chain reaction (PCR) examination of expression levels. The expressions of selected clones in selected individual fish were compared with the selection of the corresponding genes in the reference RNA. The ΔCPs were normalized by both total RNA and β-actin. *M* values were obtained from the microarray analysis of individual fish.

Accession number	Sampled fish[a]	Amplified RNA	PCR primer pair	ΔCP[b](total RNA)	ΔCP[c] (β-actin)	*M*- value	Competence between ΔCPs and *M* values
DQ849837	58-H8	Plasminogen	F: CACACAGGCCCAACATGACACC R: TTGACTCCAGGTCTGCTCGAGG	1.24	0.78	1.5	+
DQ849710	58-H8	Phospholipase A2 precursor	F: GGTTTTCGCTCCAGTTGGTCG R: CTGGACAAAGTCATGCCCGC	0.61	0.15	1.89	+
DQ849652	37-H7	Complement component C7	F: CCTGAACCAACGGCGTTTAACC R: GCTCGGAGGCGAATATCAAGCC	-3.92	-4.97	-4.14	+
DQ850124	55-H8	Apolipoprotein C-II	F: TGGTCATTTGCACCAGGGTCC R: ACCTACGCTGGCATTATGCAGG	0.41	-0.51	1.39	+ -
DQ850771	60-H8	Thioredoxin-1	F: AGCAGCCATGCCTGTTCACG R: AGCTTGTCTCCAGCCAATGCC	0.83	0.02	1.22	+ -
DQ849748	60-H8	Glutathione *S*-transferase	F: TCTCTCTGGCTGATCTGGTGGC R: CGGCCTTCAAACACATCCAGG	4.14	3.33	1.31	+
DQ850737	37-H7	β-Actin	F: ACGGACAGGTCATCACCATCG R: GGTCTCATGGATTCCGCAGG	1.05			
DQ850737	55-H8	β-Actin	As above	0.92			
DQ850737	58-H8	β-Actin	As above	0.46			
DQ850737	60-H8	β-Actin	As above	0.81			

[a]The fish designation is composed of its serial number and its assigned sample; [b]normalization by total RNA; [c]normalization by β-actin;

trend of differential expression in comparison to other samples sharing differentially expressed apolipoprotein 14kDa clones. Hence, *M* values of clones that share both annotations and similar pattern of differential expression were averaged.

After averaging, 123 differentially expressed clones remained and the number and average *M* values of up- and downregulated clones in each of the samples is presented in Table 3. Only one clone revealed differential expression in sample D7. It was the protease cathepsin L preproprotein (acc. no. DQ849956), which revealed a similar downregulation trend in all four samples. Hence, no further differential expression analysis was carried out for D7. Samples D8 and H7 revealed similar numbers of differentially expressed genes, similarly divided between down- and upregulated ones. Sample H8 revealed the highest number of differentially expressed clones composed mostly from upregulated clones. Seven clusters of differentially expressed clones were identified, A–G, presented in Table 4. A cluster contains all clones that reveal a similar *M* profile trend across fish samples. The number of clones in each clone cluster in each of the samples, divided into up- and downregulated clones are presented in Figure 1. Clusters E, F and G, containing most of the clones, were differentially expressed only in one of the samples. Hence, they are considered gene signatures of D8, H7 and H8, respectively. Interestingly, the 20 clones of cluster D elucidated resemblance between samples D8 and H8 whereas only a few clones showed resemblance between H7 and H8 (one clone), H7 and D8 (four clones) or all three samples, D8, H7 and H8 (six clones).

The 429 clones that revealed eligible *M* values in all 40 individual fish were included in the fish expression profiles which were analysed by hierarchical clustering, using three linkage procedures (Figure 2). Determination of clusters was accomplished by observing the dendrogram along its horizontal axis, from zero dissimilarity index leftward, identifying the first cluster root that contained a major number of fish from at least one sample. In case of failure to accomplish that task, the first root containing two samples was selected. If no sample-related clustering was observed reaching the upper root, no cluster was defined. All three procedures managed to cluster the *M* profiles of most fish of samples H7 and H8 and D8. The single and average linkage procedures failed to distinguish between the more similar samples D8 and H8. On the contrary, *M* profiles of sample D7 exhibited loosely clustered *M* profiles. It must be pointed out that D7 fish in Figure 2C (upper side of the dendrogram) are gradually clustered individuals at low similarity, creating no real hierarchical tree with a mutual root.

EXPANDER 5.1 permits the formation of averaged profiles of user-defined groups, followed by clustering of these groups. Hence, assembling the *M* profiles of each of the fish samples followed by their clustering, revealed similar dendrograms for all three linkage procedures, with closest D7 and D8, followed by H8 and then a remote H7 (Figure 3).

Levels of single gene biomarkers

Hepatic transcript levels of three pollutant-affected genes, metallothionein, cytochrome P4501A and ZRP were evaluated in all 40 sampled fish and the results are described in Figure 4. The two alternate normalization methods revealed similar results, hence only the β-actin normalized results are presented. The only statistically significant difference of biomarker values among samples was elucidated when testing cytochrome P4501A levels (ANOVA, F=6.144, p <0.05). However, Tamhane *post hoc* test revealed no statistically significant differences between D7 and H7 or between D8 and H8. D7 and H8 revealed a statistical difference. Metallothionein revealed no significant differences between the two sites for both years. Negligible, not significantly different ZRP levels were elucidated at both sites, at the two sampling dates.

Relative qRT-PCR validation of the microarray results

The results of relative real-time PCR, normalized to both total RNA and β-actin and compared with the respective microarray *M*-values are presented in Table 2. Similar trends of *M*-values and ΔCPs were elucidated when comparing total RNA normalized ΔCPs to *M*-values. Less stringent compatibility between ΔCPs and *M*-values was observed after normalization of ΔCPs to β-actin levels. This discrepancy was observed in clones displaying lower absolute *M* values.

Table 3. Number of differentially-expressed clones and their respective average M values ± standard deviation in each of the samples. M = $\log_2$ (normalized Cy3/normalized Cy5).

Sample	Number of upregulated clones[a]	Number of down-regulated clones[a]	Number of differentially expressed clones[a]	Average *M* value, upregulated clones	Average *M* value, downregulated clones
D7	0	1	1	0	-0.56
D8	29	16	45	0.73 ± 0.2	-0.77 ± 0.29
H7	25	21	46	0.8 ± 0.69	-0.81 ± 0.29
H8	63	6	69	0.93 ± 0.57	-0.56 ± 0.05

[a]p<0.01 and M>| 0.5 | served as thresholds for differential expression of a clone.

Table 4. Characterization of differentially expressed clones (p <0.01 and M>| 0.5|).The M values of the non-annotated sequences and also values under M>| 0.5| are available in GEO series GSE14673.

	M=[$\log_2$ normalized Cy3/Cy5]					
Cluster	M-D8	M-H7	M-H8	Accession no.	Annotation	Function
A	1.08	0.79	1.34	DQ 850431	Fructose 1,6 bisphosphate aldolase C	Generation of high-energy molecules
A	0.53	0.78	0.66	DQ 850840	Fructose-bisphosphateal-dolase B	Generation of high-energy molecules
A	0.62	0.93	1.35	DQ 849710	Phospholipase A2 precursor	Lipid metabolism
A	0.68	0.55	0.69	DQ 849958		NA
A	-1.48	-0.69	-0.62	DQ 849956	Cathepsin l preproprotein	Protease
A	-0.98	-0.80	-0.55	DQ 850270	Fibrinogen B beta polypeptide	Blood and blood clotting
B		0.51	0.96	DQ 850173	Trypsinogen	Protease
C	0.96	1.01		DQ 849837	Plasminogen	Protease
C	-0.67	-0.51		DQ 850833	Betaine-homocysteine methyl transferase	Translation-related
C	-0.70	-1.05		DQ 850742	Hepcidin-like	Iron-regulatory hormone, mediator of innate immunity
C	-0.57	-1.02		DQ 849764 DQ 849766 DQ 850667	Warm-temperature-acclimation-related-65-kDaprotein –hemopexin-like	Warm temperature acclimation and trapping of free heme for its transportation to the liver
D	0.83		0.79	DQ 850657 DQ 850312	Apolipoprotein 14 kDa	Fish lipid metabolism
D	0.71		0.66	DQ 850201 DQ 850712	Apolipoprotein C-I	Lipid metabolism
D	1.16		1.60	DQ 850174	Brain-type fatty acid-binding protein	Inflammatory
D	0.61		0.56	DQ 849865	Ceruloplasmin	Iron homeostasis oxidizer of a variety of organic substrates both anti-oxidant and pro-oxidant activities.
D	1.14		0.88	DQ 850083	Chymotrypsinogen 1-like protein	Protease
D	0.57		0.51	DQ 849667	Complement component C7	Innate immune system
D	0.65		0.69	DQ 850221	Glucose transporter 2	Transport of glucose through membranes
D	0.85		0.77	DQ 850666	Metallothionein	Metal metabolism
D	0.92		1.22	DQ 849726	Trypsinogen	Protease
D	M>0.5		M>0.5	Dq 850753 dq 850968 dq 850969 dq 849664 dq 849734 dq 849848 dq 850207 dq 850861		NA
D	-0.54		-0.51	DQ 849911	Apolipoprotein 14kDa	Fish lipid metabolism
D	M<-0.5		M<-0.5	Dq 849910 dq 851069		NA
E	0.59			DQ 850650	Beta-2 microglobulin	Component of the MHC class I
E	0.59			DQ 850880	C-type lectin	Innate immune system
E	0.79			DQ 850723	Ferritin, middle subunit	Iron storage
E	M>0.5			Dq 850888 dq 849762 dq 850210 dq 850366		NA
E	-0.63			DQ 849905	Inosine monophosphate synthase	Purine metabolism
E	-0.61			DQ 849733	Apolipoprotein A-I precursor	Lipid metabolism
E	-0.71			DQ 850062 DQ 850140	Complement component C3	Innate immune system
E	-1.26			DQ 850516	Complement component factor H	Innate immune system
E	-0.53			DQ 887347	Dab2	
E	-0.91			DQ 850289	Elongation factor 1-alpha	Delivery of aminoacyltRNAs to the ribosome.
E	-0.57			DQ 850060	Transferrin	Iron transporter
E	M<-0.5			DQ 851081		NA
F		0.60		DQ 850539	Apolipoprotein A-I	Lipid metabolism

Table 4. continued on next page

Table 4. Continued.

	M=[$\log_2$ normalized Cy3/Cy5]					
Cluster	M-D8	M-H7	M-H8	Accession no.	Annotation	Function
F		0.73		DQ 849703 DQ 850977	Ceruloplasmin	Iron homeostasis oxidizer of a variety of organic substrates both anti-oxidant and pro-oxidant activities.
F		0.53		DQ 850924	Collagen XVIII alpha 1 chain	Structural component of the basal lamina
F		0.65		DQ 850117	Cytochrome P450 4H16	Oxidative stress
F		0.63		DQ 850188	Elongation factor 1-alpha	Delivery of aminoacyltRNAs to the ribosome.
F		0.50		DQ 850163	Glutathione peroxidase	Oxidative stress
F		0.51		DQ 850232	Liver-basic fatty acid binding protein	Lipid metabolism
F		0.55		DQ 850115	Peroxiredoxin 6	Oxidative stress
F		0.51		DQ 850052	Transcobalamin-2	Vitamin transportation
F		M>0.5		Dq 850932 dq 850944 dq 850171 dq 850084 dq 851046 dq 851064 dq 849979 dq 849828 dq 849728 dq 850133		NA
F		-1.30		DQ 850682	Apolipoprotein E	Lipid metabolism
F		-0.71		DQ 850732	Catalase	Oxidative stress
F		-0.71		DQ 849636 DQ 850927	Chemotaxin	Chemotaxis
F		-1.71		DQ 849652	Complement component C7	Innate immune system
F		-0.58		DQ 849860	Cytochrome C oxidase subunit 1	Generation of high-energy molecules
F		-0.55		DQ 850127	Endothelin receptor A	Signal transduction
F		-0.54		DQ 849770	Fibrinogen gamma polypeptide	Blood and blood clotting
F		-0.89		DQ 849783	Hemoglobin beta-A chain	Blood and blood clotting
F		-0.51		DQ 850860	Hepcidin-like	Iron-regulatory hormone mediator of innate immunity
F		-0.79		DQ 850950	Membrane-spanning 4-domains subfamily A member 15	Putative receptor activity signal transduction
F		-0.65		DQ 850006	N-myristoyl transferase 1	Adhesion to membranes
F		-0.73		DQ 850697	Rho family, small GTP binding protein Rac 1	Signal transduction
F		M<-0.5		Dq 850013 dq 849674 dq 850001 dq 851067		NA
G			0.53	DQ 850903	Apolipoprotein 14 kDa	Fish lipid metabolism
G			0.74	DQ 850124	Apolipoprotein C-II	Lipid metabolism
G			0.95	DQ 849717	Bone morphogenetic protein 1B	Ossification
G			0.63	DQ 850260	Cathepsin L	Protease
G			1.42	DQ 849713	Ceruloplasmin	Iron homeostasis oxidizer of a variety of organic substrates. Reveals anti-oxidant and pro-oxidant activities.
G			0.54	DQ 849641	Coagulation factor II	Blood and blood clotting
G			0.56	DQ 850854	Complement component C3	Innate immune system
G			0.54	DQ 849704	Fibronectin 1B	Blood clotting
G			1.27	DQ 849771	Fructose-bisphosphate aldolase B	Generation of high-energy molecules
G			0.60	DQ 849748	Glutathione *S*-transferase, Theta 3	Detoxification of xenobiotics
G			0.86	DQ 850976	Hemeoxygenase	Oxidative stress
G			1.01	DQ 849644	Hepcidin 5	Iron-regulatory hormone mediator of innate immunity
G			1.29	DQ 850852	Insulin-like growth factor binding protein 1	Controlling IGF
G			1.20	DQ 850200	Membrane associated progesterone receptor	Reproduction

Table 4. continued on next page

Table 4. Continued.

	M=[log$_2$ normalized Cy3/Cy5]					
Cluster	M-D8	M-H7	M-H8	Accession no.	Annotation	Function
G			3.60	DQ 850845	Phospholipase A2 precursor	Lipid metabolism
G			0.72	DQ 849891	Ribosomal protein-40s S26	Translation
G			0.60	DQ 850979	Ribosomal protein-40s S27	Translation
G			0.78	DQ 849767	Ribosomal protein-60s l11	Translation
G			0.66	DQ 850507	Techylectin	Putatively involved in innate immunity
G			0.53	DQ 850771	Thioredoxin-1	Oxidative stress
G			0.89	DQ 849715	Trypsinogen 7 isoform 3	Protease
G			M>0.5	Dq 850106 dq 850638 dq 850639 dq 850972 dq 850314 dq 850326 dq 849723 dq 849727 dq 849738 dq 849774 dq 849808 dq 849859 dq 850009 dq 850023 dq 850034 dq 850693 dq 850837 dq 850951 dq 850865 dq 849868		NA
G			M<-0.5	Dq 850602		NA

Alternate grey-highlighted and white rows designate different gene clusters (A–G). D8, H7 and H8 are sampling sites designations. NA, no available annotation.

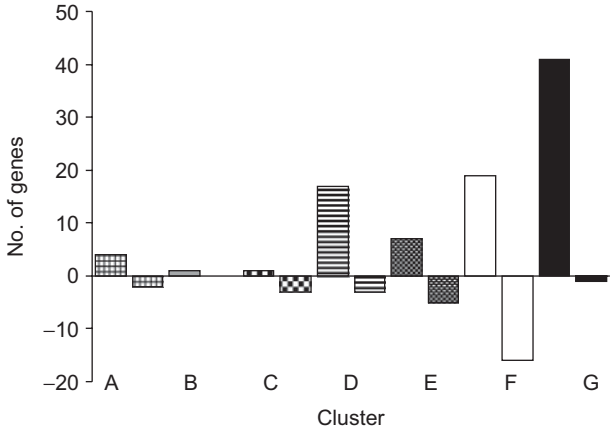

Figure 1. *Lithognathus mormyrus* sample-related numbers of up- or downregulated clones elucidated by clusters A–G (Table 4), selected using p <0.01 and M>| 0.5| as thresholds.

Discussion

The microarray results were validated by the qRT-PCR when using total RNA as the normalizing agent. When using β-actin for normalization, the results were less compatible. The direction of expression in relation to the reference had been reversed for one of the examined clones, and was closer to ΔCP=0 in another. This discrepancy may be explained by the different normalization factors used in the two analytical procedures, which could result in different *M* direction of mildly differentially expressed clones.

The relationship between fish samples and gene expression-related features was examined using three tools, differential gene expression in each of the samples, clustering of fish gene expression profiles and gene annotations. Differential expression was mainly elucidated in H7, D8 and H8 with only one differentially expressed gene in D7. The lack of differential expression in D7 makes sense, as the equally combined RNA of D7 was taken as a reference RNA. Therefore, the differential expression results can serve for analysing only samples D8, H7 and H8. The selection of D7 fish as the present source for reference RNA and not a mixture of all participating fish was motivated by the rationale of selecting a group of fish, assumed to be exposed to a uniform low pollution pressure.

The compatibility of the working assumptions (see Introduction above) with the analysed data was quite firmly demonstrated. The indicating evidence includes the formation of reasonable sample-related clusters of *M* profiles of individual fish for three of the samples (Figure 2). In addition, three sample-specific gene signatures were observed, clone clusters E, F and G (Table 4, Figure 1) characterizing samples D8, H7 and H8, respectively. These three clone clusters include most of the differentially expressed genes, 89 out of 123.

The unexpected difference between H7 and H8 may be explained by variable pollution composition and intensity at the Haifa site due to intermittent drainage from the adjacent polluted Kishon River and due to occasional dredging activities in the harbour region, causing resuspension and/or removal of polluted sediment. In addition, the length of the sampled fish (Table 1) indicated sampling of 1-year-old specimens throughout this study. The age was deduced from previously evaluated length-to-age relationships (Kallianiotis et al. 2005). This short lifespan may also contribute to the difference between H7 and H8, as the putative temporally different environmental impact affected different fish populations, with no accumulatedinfluence of the previous year.

The relative similarity between D8 and H8 was demonstrated by the failure of two clustering procedures to distinguish between them (Figure 2) and by the partial similarity of differential expression (clone cluster D, Table 4). It is difficult to explain this similarity. It may be speculated that certain environmental parameters were common along the Israeli coast of the Mediterranean

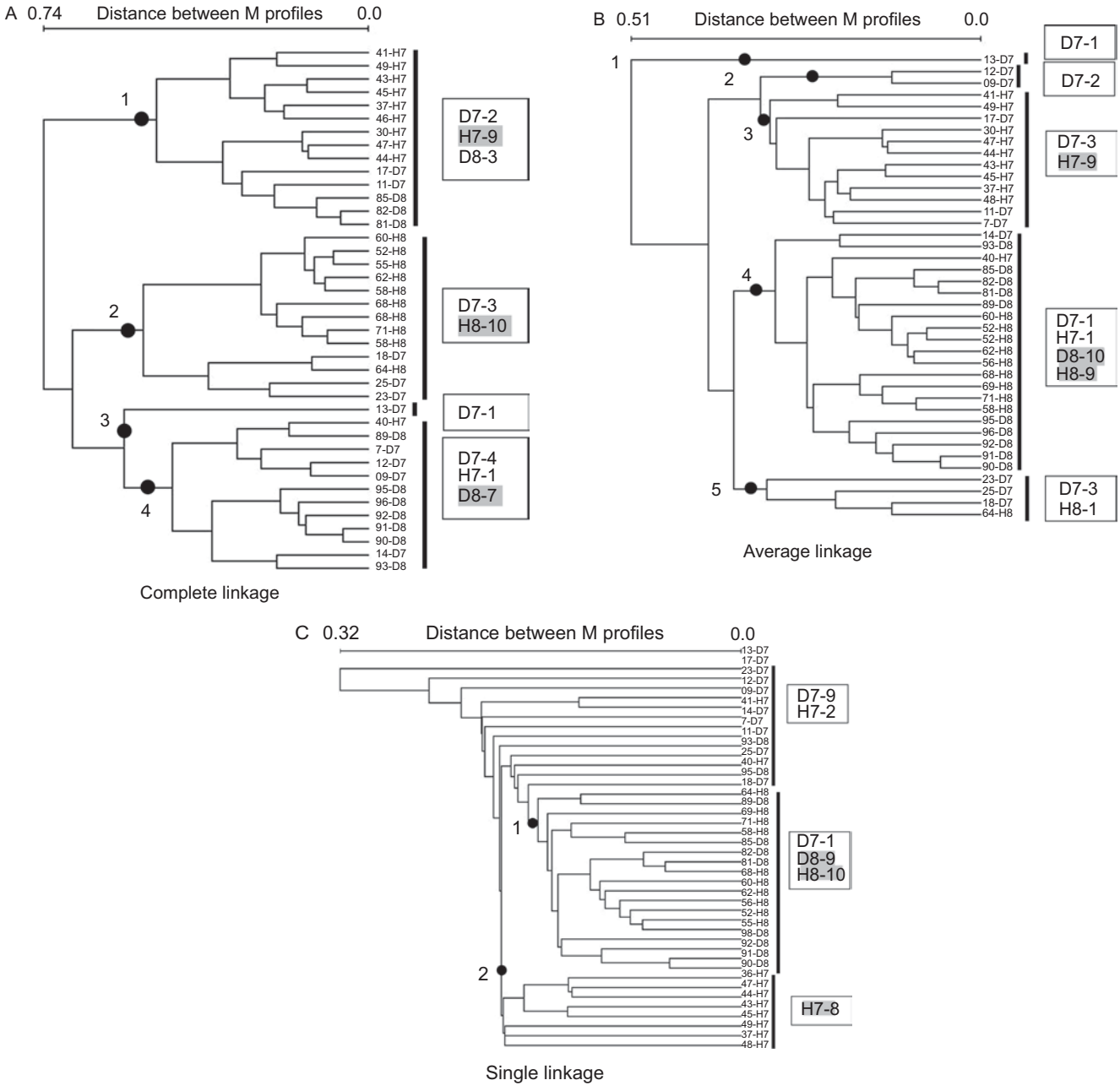

Figure 2. Hierarchical cluster analysis of *M*-value profiles of individual *Lithognathus mormyrus* according to three clustering procedures (A-C). *y*-Axis ranges 0–1, zero represents complete similarity; H, Haifa site; D, Dor site; 7-, sampled in 2007; 8-, sampled in 2008. Defined clusters are numbered and labelled by black vertical lines. Black dots identify the root of each cluster tree. The number of fish from each sample in each cluster is depicted within frames, right of each cluster. A major sample in a cluster is highlighted in grey.

in the winter of 2008. It was not possible to compare D7 and H7 as no differential expression was observed in D7 fish (Table 3) as well as coherent clustering of their clone expression profiles (Figure 2).

When combining *M* profiles of individual fish into sample-related profiles through EXPANDER 5.1, D7 and D8 elucidated the highest similarity, with gradually increasing distance from H8 and H7 at the respective order. This high similarity may be explained by the identical sampling site, compatible with the assumed permanent low pollution pressure at the Dor site expected to exhibit similar gene expression patterns. However, this similarity is compromised by the distinction between D7 and D8, demonstrated by the D8 specific gene cluster E, and also by the partial similarity of D8 to H8, demonstrated by gene cluster D.

Relating the elucidated *M* profiles to specific samples leads to the issue of the extent of their relevance to pollution pressure. The key factor is an adequate reference RNA that should be extracted from fish that were never

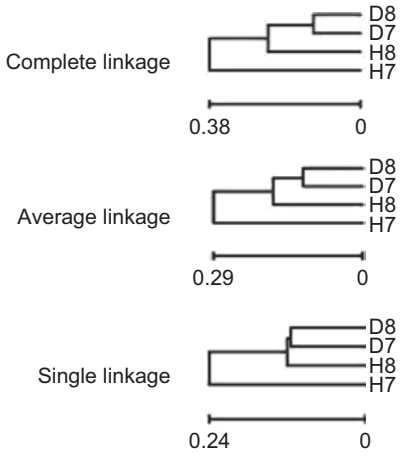

Figure 3. Hierarchical cluster analysis of *M*-value profiles of the four fish samples, carried out according to three clustering procedures. *x*-Axis ranges 0–1, zero represents complete similarity; H, Haifa site; D, Dor site; 7-, sampled in 2007; 8, sampled in 2008.

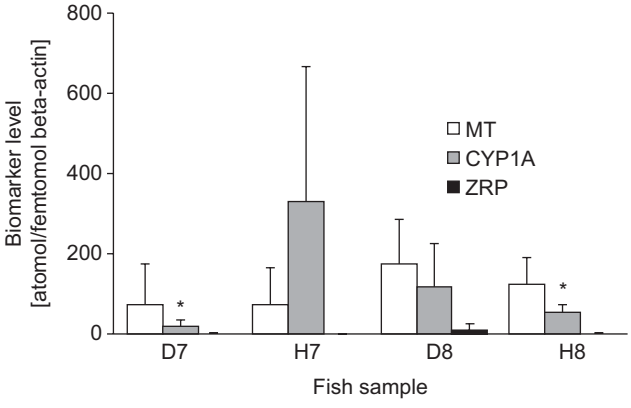

Figure 4. Hepatic levels ofmetallothionein (MT), cytochrome P4501A (CYP1A) and zonaradiataprotein (ZRP) in the *Lithognathusmormyrus*samples.The bars represent atomol transcript/femtomolβ-actin and the error bars represents their standard deviations. One-way ANOVA for each of the three transcript biomarkers among the defined groups revealed no differences for MT (df=2, F=2.61 and p >0.05) and ZPR (df=2, F=1.336 and p >0.05). CYP1A revealed significant differences (df=2, F=6.144 and p <0.05). Tamhane*post hoc* tests revealed significant difference only between D7 and H8 (*p <0.05).

exposed to pollution, belonging also to a uniform population with minimal pollution-independent changes among their gene expression profiles. Consequently, the present reference mixture which was taken from D7 could not be considered a hallmark of fish unexposed to pollution as it was not sufficiently examined. However, the similarity between D7 and D8 and their marked difference from H7 and H8 (Figure 3) may initially indicate their adequacy to serve as a reference.

The difficulty of determining a solid reference RNA leads to suggesting a sampling effortover several years, basically an improved version of the present study, aimed at defining an appropriate reference. The improvements must include: (1) monitoring the population dynamics using molecular markers, such as the tools used by Sala-Bozano et al. (2009) studying *L. mormyrus* populations;(2) auxiliary monitoring of biomarkers of a different nature (e.g. cellular, physiological); and also (3) a more comprehensive chemical monitoring of pollutants, even if not covering the entire potential pollutant spectrum.

Hence, multi-annually sampled fish belonging to a uniform population, caught in a site that was extensively tested for low pollution pressure would be considered candidates for a source for a multi-annual reference RNA. Intimate clustering of their *M* profiles would strongly indicate their adequacy as contributors to a reference RNA. It is a complicated solution but there is no way to circumvent it considering the complex biomarker, which is affected by a variety of pollution-dependent and -independent parameters.

Falciani et al. (2008), using a flounder cDNA microarray applied similar approach to ours. They examined hepatic expression profiles of flounders sampled from several polluted and non-polluted sites. Unlike the present study, their within-sample clustering of individual *M* profiles revealed only a partial relationship to the sampling sites. Hence, they postulated a potential masking of environment-affected gene expression profiles by patterns related to individual variability and to pollution-independent biological factors. Consequently, using their developed software GALGO, they searched for subsets of genes, assumedly pollution-affected, relating them to the different sets of exposure conditions. Such signatures were not found automatically by GALGO as initially expected, but only when selecting a subset of genes, already indicated in the literature to be affected by pollution.

Genes of clusters F and G uniquely characterized the two Haifa samples, and may be initially considered pollution affected, being signatures of assumedly polluted site. Hence, it would be interesting to compare their expression patterns with previous pollution exposure studies. A word of caution to be put forward here is that conflicting differential expression directions of specific genes between the present study and the compared experimental exposures may result from a real different biological situation, but also from different utilized reference or control RNA. Two exposure experiments of *L. mormyrus* to xenobiotics were performed recently using the present microarray. Exposure to cadmium was presented in the study by Auslander et al. (2008) and exposure to the pro-oxidant *tert* butyl hydroperoxide in Auslander et al. (2010). Compared differential gene expression among the three studies is presented in Table 5. In addition to the functional data presented

Table 5. The direction of hepatic differential expressions of genes of clusters F and G in the present study compared to *L. mormyrus* exposure to cadmium (Auslander et al. 2008) and *tert* butyl hyderoperoxide (tBHP; Auslander et al. 2010). Up or down designate expression directions.

	Direction of differential expression		
Gene name	Present study	Cadmium exposure	tBHP exposure
14 kDa Apolipoprotein	Up		Up
Apolipoprotein C-II	Down		Up
Apolipoprotein E	Down		Up
Ceruloplasmin	Up	Up	Up
Complement component C3	Up		Down
Complement component C7	Down		Down
Cytochrome C oxidase	Down	Up	Up
Fibrinogen gamma polypeptide	Down		Down
Fructose-bisphosphate aldolase B	Up or Down		Up
Glutathione peroxidase	Up	Up	
Hemeoxygenase	Up		Up
Hemoglobin beta-A chain	Up or Down		Up
Hepcidin	Down		Down
Membrane-spanning 4-domains subfamily a member 15	Down		Down
Rho family-small GTP binding protein Rac 1	Down		Down

in Tables 4 and 5, comments related to genes of clusters F and G are followed below, further indicating their relationship to environmental stress parameters.

Glutathione peroxidase which reduces lipid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water, was upregulated upon exposure of carp to cadmium (Reynderset al. 2006) and upon flounder exposure to pro-oxidants (Williams et al. 2008). Cadmium is known to contribute to oxidative stress (Halliwell&Gutteridge 1999), hence, may affect glutathione peroxidase levels. Ceruloplasmin is a multifunctional protein, which besides its role in iron metabolism is involved in response to inflammation, and functions as both anti- and pro-oxidant (Bielli&Calabrese 2002,Hellman &Gitlin 2002). It was upregulated in the fish *Sebastesschlegeli* in response to benzo(a)pyrene application (Yum et al. 2006). Hepcidin is a negative mediator hormone of iron absorption and its release from macrophages is suppressed by anemia or hypoxia (Ganz 2003). It was downregulated in the study by Williams et al. (2007) upon exposure to estrogen and in Williams et al. (2008) as an acute response to cadmium, lindane and perfluorooctanoic acid applications. However, in the later study, 16 days after exposure it reversed its expression trend and was upregulated. Hemeoxygenase is the first enzyme in the heme catabolic pathway, producing the catabolytesbiliverdin, iron and carbon monooxide. It was induced during oxidative stress (Kikuchi et al. 2005). Peroxiredoxin and thioredoxin were induced in the study by Koskinen et al. (2004) upon exposure to β-naphthoflavone and in Williams et al. (2008) in response to exposure to several pro-oxidants. Complement components C3 and C7 were downregulated in Yum et al. (2006) in response to benzo(a)pyrene exposure.Glutathione-*S*-transferase, belonging to a xenobiotic phase II metabolism group of enzymes was induced by a variety of organic pollutants in Williams et al. (2008). The presented functional data of differentially expressed genes and their differential expression in a variety of exposure experiments further indicates the suitability of the examined tool to serve as an environmental biomarker.

The parallel evaluation of three established stand-alone biomarker genes demonstrated clearly that much more information and delicate distinction among sites and dates of sampling could be obtained by the comprehensive transcriptomic approach. A difference between cytochrome P4501A levels in the Haifa and Dor sites evaluated by the absolute qRT-PCR, was demonstrated in the years 2004–5 (Yudkovskiet al. 2008). Different levels, but not statistically significant forcytochrome P4501A were revealed here between D7 and H7. The lack of statistical significance was due to the high variability of the results. D8 and H8 revealed similar levels of cytochrome P4501A transcript, data which have poor biological relevance. On the array, cytochrome P4501A (acc. no.DQ850233) elucidated eligible spots only in H7 and H8 and both were not significantly different from M=0. No difference was observed among the metallothionein transcript levels of the four samples, the same as in the microarray results. ZRP spots on the array revealed a weak signal (see GEO GSE2262) and the M values could not be compared with its transcript levels evaluated by qRT-PCR.

In conclusion, a pilot utilization of a transcriptome expression profile-based monitoring system was examined. Its suitability for biomonitoring was evaluated, revealing also its weaker aspects which need improvement. The analysis distinguished between annual

samples of the polluted site, indicating variable levels and composition of pollution. The fish samples from the assumedly clean site revealed similarity between them over time, indicating similar pollution pressure. The utilized biomarker parameters provided better resolution among sampled sites than the parallel evaluation of stand-alone biomarker genes. However, the study pointed out the need for a reference RNA credibly originating from individuals not exposed to pollution, to provide stronger relationships between differential expression and environmental effects.

More sensitive and accurate microarray platforms demonstrating lower background have been recently introduced to the microarray arsenal. Consequently, applying the presented analytical tools using a more comprehensive gene assemblage produced by advanced molecular methods (e.g. pyro-sequencing coupled to sequence-based oligo-array) may improve resolution by revealing more differentially expressed genes with annotated functions. These more informative expression profiles would lead to more educated interpretation of the results.

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Declaration of interest

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