Comparison of the level of organic contaminants in bile and muscle of *Mugil* spp. following a major oil spill in the eastern Mediterranean Sea

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Summary

The total polyaromatic hydrocarbons (TPAH) and the total polychlorinated biphenyls (TPCB) that originate from oil spills in sea and ocean waters are toxic to fish and their offspring. The authors compare the levels of organic contaminants (TPAH and TPCB) recovered from the bile versus the dorsal muscles of 120 individual *Mugil* spp. that were harvested from six sites in the eastern Mediterranean following a significant heavy oil spill. Results showed an insignificant difference between the mean of means of TPAH and TPCB (six means of individual *Mugil* spp. from six respective sites) in bile versus dorsal muscle. In addition, the correlation equation between the level of bile TPAH and the level of bile carcinogenic polyaromatic hydrocarbons (cPAH) was established. This data suggests the possibility of substituting the analysis of organic contaminants in muscles by using the liquid bile of *Mugil* spp., thus eliminating the time-consuming steps of lyophilisation and homogenisation of muscle. In addition, the bile cPAH could be predicted from the bile TPAH by a regression relationship.

Keywords

Bile, Carcinogenic polyaromatic hydrocarbon, Correlation, cPAH, Dorsal muscle, Lebanon, Mediterranean, *Mugil* spp., PAH, Total polyaromatic hydrocarbon, Total polychlorinated biphenyl, TPAH, TPCB.

Confronto della concentrazione di contaminanti organici in bile e tessuto muscolare di *Mugil* spp., in seguito a ingente sversamento di petrolio nel Mar Mediterraneo orientale

Riassunto

Gli idrocarburi poliaromatici totali (TPAH) e i bifenili policlorurati totali (TPCB) derivanti da sversamenti di petrolio nelle acque del mare e dell'oceano sono tossici per la popolazione ittica e la loro progenie. Questo studio mette a confronto i livelli di contaminanti organici (TPAH e TPCB) rilevati in bile e muscoli dorsali di 120 individui di Mugil *spp., prelevati in sei punti del Mediterraneo orientale in sequito alla fuoriuscita ingente di petrolio. I risultati hanno evidenziato una differenza irrilevante tra la media delle medie dei valori di TPAH e PTCB (sei medie di individui di* Mugil *spp. da sei differenti punti di prelievo) riscontrati nella bile rispetto ai muscoli dorsali. È stata inoltre calcolata un'equazione di correlazione tra i livelli di TPAH e PAH carcinogenico (cPAH) nella bile. I dati suggeriscono la possibilità di sostituire l'analisi dei contaminanti organici nei muscoli con la bile liquida di* Mugil *spp.,*

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eliminando di conseguenza le fasi di liofilizzazione e omogeneizzazione muscolare che richiedono molto tempo. Inoltre mediante relazione di regressione, si potrebbe predire il livello di cPAH nella bile sulla base del livello di TPAH nella bile stessa.

Parole chiave
Bifenile policlorurato totale, Bile, cPAH, Correlazione, Idrocarburo poliaromatico carcinogenico, Idrocarburo poliaromatico totale, Libano, Mediterraneo, *Mugil* spp., Muscolo dorsale, PAH, TPAH, TPCB.

Introduction

The oil spills in seas and oceans that occur around the world deliver a significant amount of organic compounds to the environment (5), some are hazardous to the biological systems of marine organisms, as is the case with many members of the polyaromatic hydrocarbon (PAH) and of the polychlorinated biphenyl (PCB) families (8, 14).

The documented health hazards of such compounds on marine life and in certain instances on humans (14, 15) requires continuous monitoring of the total PAH and PCB in selected marine organisms especially following significant oil spills into seas or oceans (3, 12). *Mugil* spp. are very common fish in the eastern Mediterranean Sea, and their behaviour has been studied extensively, confirming their non-repulsive nature to apparent accumulating oil in marine waters (10). This fact makes the individuals of the *Mugil* spp. an appropriate indicator fish that accumulates organic compounds in its body as a result of oil spills (2).

The universal testing of the accumulated organic compounds, originating from an oil spill is usually accomplished on the muscle of fish that are consumed by humans. The use of the muscle requires time-consuming steps of lyophilisation and homogenisation, before the steps of total PAH (TPAH) and total PCB (TPCB) extraction. In addition, many samples of fish are required and sampling of the muscle will lead to losses to the fishermen present in many parts of the world. Literature has shown that the enzymatic activities in the liver of fish cannot metabolise such organic compounds completely, thus leading to their presence in the bile (1, 14). In addition, previous works have shown that some TPAH compounds can be processed in the liver of the fish, producing more health hazardous carcinogenic PAH (cPAH) metabolites that are secreted in the bile and then reabsorbed in the fish intestines and accumulating back in the muscle (3).

These sporadic reports in the literature helped to construct the objective of this work which aims to correlate the levels of TPAH and the TPCB in dorsal muscle versus their levels in bile of *Mugil* spp., in an attempt to determine if the analysis of these organic compounds in liquid bile could, in the future, replace the analysis of dorsal muscle. If successful, this attempt is expected to save time needed for lyophilisation and homogenisation of muscle and avoid economic losses caused by the generous use of fish muscle in sampling. Additional work is included in this research project which attempts to estimate the bile cPAH from the bile TPAH levels in the harvested *Mugil* spp. individuals.

Materials and methods

Location of the study

Six sites across the eastern Mediterranean shores of the Lebanon, located at different distances to the South (24.1-47.8 km) and to the North (30.3-78.3 km) of the source of the oil spill were selected for harvesting *Mugil* spp. individuals. The coordinates of the six sites, named by the coastal towns or cities and in accordance with global positioning system (GPS) data provided by the Greenpeace divers of the *Rainbow Warrior*, were as follows:

- Tyr (N 33°17.548′, E 035° 12.741′)
- Sarafand (N 33° 28.388′, E 035° 16.749′)
- Beirut (N 33° 53.579′, E 035° 27.855′)
- Tabarja (N 34° 01.089′, E 035° 37.264′)
- Amchit (N 34° 07.729′, E 035° 38.209′)
- Barbara (N 34° 12.181′, E 035° 38.060′).

Mugil spp. harvests

Mugil spp., a predominant species of fish in the traditional fishing activities of the Lebanon, were sampled from the six locations mentioned above. A total of 120 fish of *Mugil*

spp. were collected from the six locations (20 fish/location). The sampling of the fish took place 117 days after the spill of 15 000 tons of heavy fuel oil into the eastern Mediterranean Sea. The average length of fish caught was 31.2 cm. Sexing of harvested fish was not attempted.

Muscle versus bile collection

The dorsal muscle was removed aseptically and stored in a sterile container, while the bile was collected from the bile duct located beneath the liver, using sterile needles and syringes. The muscle and bile samples were stored at –80°C and –20°C, respectively, until analysed. It is worth noting that the bile metabolite concentrations were not correlated to the digestive status, since the fish were harvested at dawn, a time when the fish were actively feeding.

Differences in muscle versus bile treatment

The solid muscle samples were individually homogenised by a tissue grinder and lyophilised at –50°C and 0.22 mBar pressure for 48 h (9), and then rehomogenised and stored at –80°C. The liquid bile samples were not subjected to any homogenisation or lyophilisation and were stored at –20°C.

Extraction of polyaromatic hydrocarbons, polychlorinated biphenyls and carcinogenic polyaromatic hydrocarbons

Each 0.5 g of a lyophilised and homogenised dorsal muscle and each 0.5 ml of a bile sample was suspended in 2.5 ml of high-performance liquid chromatography (HPLC) grade methanol (1/5 w/v). The reconstituted samples were then vortexed for 3 min and centrifuged for 10 min at 2 000 rpm to extract the PAH, PCB and cPAH into the methanol phase (11). Three respective standard curves were established for PAH, PCB and cPAH correlating different levels of each (parts per million: ppm) to optical density (OD) values, using the rapid assay test kits developed by Strategic Diagnostics Inc. in 1997 (13) and approved by the United States Environmental Protection Agency (2). The three respective standard curves are shown in Figures 1, 2 and 3.

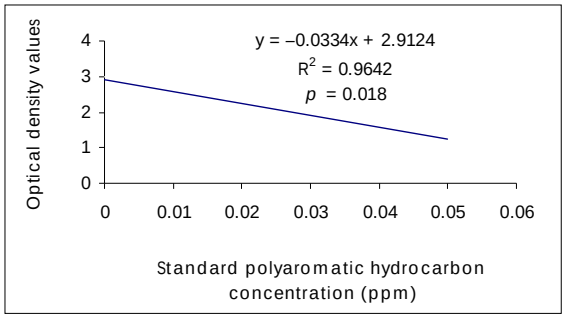

Figure 1
Line of best fit correlating the standard polyaromatic hydrocarbon concentration (ppm) to optical density

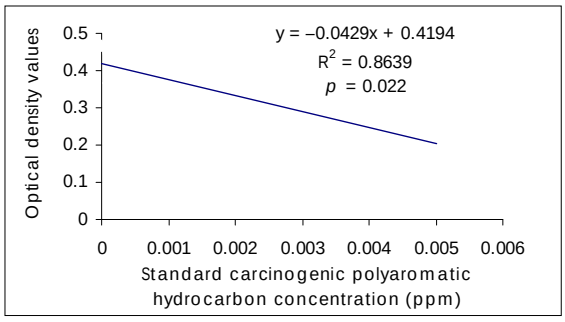

Figure 2
Line of best-fit correlating the standard carcinogenic polyaromatic hydrocarbon concentration (ppm) to optical density

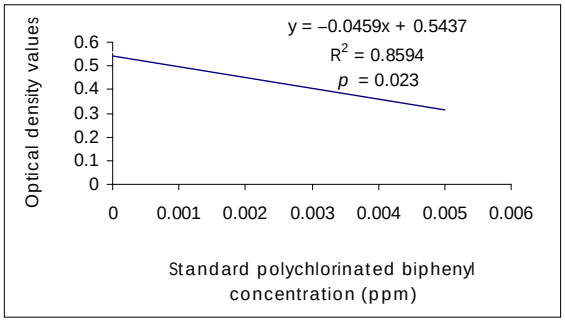

Figure 3
Line of best-fit correlating the standard polychlorinated biphenyl concentration (ppm) to optical density

Analysis of polyaromatic hydrocarbons, polychlorinated biphenyls and carcinogenic polyaromatic hydrocarbons

The analysis of methanol extracted-PAH, PCB and cPAH was performed using three different rapid enzymatic assay test kits (Strategic Diagnostics Inc., Newark, Delaware). Briefly, each of the three analyses required a volume of 250 µl of the methanol extracts of the fish samples, or standards, or controls, in which each was added to a polystyrene test tube. The determination of PAH, PCB and cPAH required the addition of 250 µl of the respective conjugates of enzyme-PAH, enzyme-PCB, enzyme-cPAH, respectively. After thorough mixing, a volume of 500 µl of magnetic particles coated with antibodies specific to the respective total PAH, TPCB, or total cPAH compounds were added. These coated antibodies compete for either the TPAH, TPCB or total cPAH carried by the conjugates, and for TPAH, TPCB or total cPAH present in the standards, controls, or in the methanol extracts of fish samples. The mixture was incubated for 30 min at room temperature. A magnet was then placed at the bottom of the tube rack to attract the reactants to the bottom of the tubes. The supernatants were discarded and the magnetically attracted reactants were washed twice using 1 ml of a washing solution provided by the kit, followed by the addition of 500 µl of a substrate and an incubation period of 20 min. An amount of 500 µl of a stop solution was then added to terminate the enzymatic reaction and the ODs of the developed colours were read at a wavelength of 450 nm, using a spectrophotometer (Turner Spectrophotometer SP-890, UV-VIS, Dubuque, Iowa).

The OD reading for standards, controls and methanol extracts for each of the three organic chemical groups namely: PAH, PCB and cPAH were referred to the three respective standard curves (Figs 1, 2 and 3) to determine their levels.

The respective range of detection limits of the PAH, PCB and cPAH are 0.00135-0.4325 ppm, 0.001-0.0677 ppm and 0.0002-0.010 ppm (Strategic Diagnostics Inc.).

Statistical analysis

The mean of PAH and PCB, in muscle and bile of *Mugil* spp. harvested at each of the six sites was obtained (20 samples of fish/site). The mean of means for the PAH in muscle ($n = 120$) versus bile samples ($n = 120$) were compared at $p = 0.05$ by one-way analyses of variance (ANOVA), followed by Duncans test (SPSS 15.0, SPSS Inc., Chicago, Illinois), reporting the standard errors (SE) and the standard error of means (SEM). The same statistical procedure was used to compare the mean of means of PCB in muscle ($n = 120$) versus PCB in bile samples ($n = 120$). In addition, the means of cPAH in bile were compared among *Mugil* spp. organisms harvested from each of the six sites ($n = 20$ from each site). Linear regression equations were established for PAH, PCB and cPAH in relation to OD values at $p < 0.05$ and to correlate the TPAH to the total cPAH in bile samples at a $p = 0.1$.

Results

The three respective standard curves correlating the levels of PAH, PCB, cPAH to OD values are shown in Figures 1, 2 and 3. The three regression equations were statistically significant in their correlations at $p < 0.05$. The R^2 values for the three respective standard curves of PAH, PCB and cPAH were: 0.9642, 0.8594 and 0.8639. All three standard curves had negative slopes, indicating an inverse relationship between the contaminant concentration and the OD value.

The comparison of the means of the TPAH in bile versus dorsal muscle of *Mugil* spp. collected from the six sites located at different distances from the source of the oil spill is shown in Table I. The mean of the means of the total PAH revealed no statistical difference when analysed in bile samples versus that in the dorsal muscle samples (0.302 versus 0.238 ppm, respectively) ($p > 0.05$). The PAH data in muscle and the PAH standard curve establishment were reported by Barbour *et al.* in 2008 (2).

Table II shows the data comparing the means of the TPCB in bile versus dorsal muscle of *Mugil* spp. collected from the six sites. The mean of means of the TPCB in bile samples versus that in the dorsal muscles did not differ significantly (0.025 versus 0.033 ppm, respectively) (p >0.05).

The means of the total bile CPAH in *Mugil* spp. harvested from the six sites ranged between 0.011 ppm and 0.025 ppm (Fig. 4). No statistical difference was observed among the six means of the total bile cPAH obtained from the *Mugil* spp. harvested from the six sites (p >0.05). The bile TPAH level correlated significantly to the bile cPAH in *Mugil* spp. at a confidence level of 90%, resulting in a positive slope regression equation of Y (bile TPAH) = 0.0057X (bile total cPAH) + 0.21.

Discussion

The negative slopes and the R^2 values shown in Figures 1, 2 and 3 confirm the concept of the competitive rapid enzymatic systems that were used in this study (13), allowing the quantitation of the PAH, PCB and cPAH parameters in the different samples collected.

Table I
Comparison of total polyaromatic hydrocarbons in bile versus dorsal muscle of *Mugil* spp. collected from six sites along the eastern shores of the Mediterranean

Location		Mean of total polyaromatic hydrocarbons (ppm) ± SE	
Shore sites	Distance from oil source (km)	Bile	Dorsal muscle
Tyr	47.8 S	0.354±0.012	0.096±0.069
Sarafand	24.1 S	0.339±0.027	0.108±0.070
Beirut	30.3 N	0.251±0.091	0.349±0.117
Tabarja	57.9 N	0.292±0.109	0.380±0.064
Amchit	72.4 N	0.234±0.162	0.244±0.094
Barbara	78.3 N	0.345±0.196	0.252±0.098
Mean of means± SEM		0.302*±0.044	0.238*±0.039

ppm parts per million
SE standard error
SEM standard error of means
* mean of means of total polyaromatic hydrocarbon levels in bile and dorsal muscle were insignificantly different (p >0.05)

Table II
Comparison of total polychlorinated biphenyls (PCB) in bile versus dorsal muscle of *Mugil* spp. collected from six sites along the eastern shores of the Mediterranean

Location		Mean of total polychlorinated biphenyls (ppm) ± SE	
Shore sites	Distance from oil source (km)	Bile	Dorsal muscle
Tyr	47.8 S	0.0256±0.009	0.033±0.011
Sarafand	24.1 S	0.018±0.012	0.044±0.002
Beirut	30.3 N	0.025±0.014	0.036±0.012
Tabarja	57.9 N	0.016±0.009	0.027±0.009
Amchit	72.4 N	0.042±0.009	0.014±0.005
Barbara	78.3 N	0.024±0.014	0.044±0.007
Mean of means± SEM		0.025*±0.005	0.033*±0.004

ppm parts per million
SE standard error
SEM standard error of means
* mean of means of total polyaromatic hydrocarbon levels in bile and dorsal muscle were insignificantly different (p >0.05)

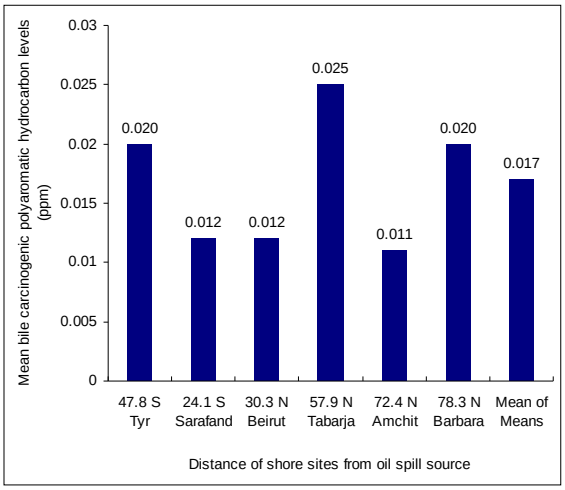

Figure 4
A comparison of the bile carcinogenic polyaromatic hydrocarbon level (ppm) in *Mugil* spp. collected from the six sites

The lack of data on the sex of the *Mugil* spp. fish could affect the dynamics of relatedness between the levels of the parameters measured in muscle versus bile samples. Future investigations should establish such relatedness in male versus female *Mugil* spp. The statistically similar mean of means of TPAH in muscle and bile of *Mugil* spp. (Table I) (p >0.05) is mainly due to the fat-soluble nature of TPAH compounds (7), in which the mobilised fat, associated with TPAH, is released from the muscle, metabolised by the mixed function enzyme systems present in the liver and later converted into lipophilic products emulsified with water, thus accelerating their excretion with the TPAH into the bile (8). The insignificant difference in the mean of the means of TPAH in the muscle and in the bile indicates that the metabolism conserved the main backbone chemical structure of the PAH compounds, which is a common antigenic determinant that can be detected by the specific antibodies used in the competitive enzymatic system. Harvesting of the fish 117 days after the oil spill could have provided sufficient time for the PAH to correlate in bile and muscle. Future investigations should include a laboratory experiment to demonstrate the hypothesis of the correlation of PAH in muscle and bile, as affected by the time variable, following the exposure to a known amount of PAH in such heavy oil.

The PCBs are also fat-soluble compounds (6) that could have been mobilised from the adipose tissue and metabolised in the liver in dynamics that are similar to the PAH compounds, thus resulting in an insignificant difference in the mean of the means of TPCB in the muscle and in the bile (Table II).

The range of the cPAH in the bile of *Mugil* spp. was between 0.011 ppm and 0.025 ppm (Fig. 4). The level of cPAH in the bile of other fish has been reported in the literature (4). In the bile of the English sole (*Pleuronectes vetulus*), the range of a marker of exposure to cPAH is reported to be between 0.0158 ppm and 0.054 ppm. The range of the marker in the bile of the same fish harvested from another site was between 0.0169 ppm and 0.0395 ppm (4). This data is in agreement with the data obtained for the bile of *Mugil* spp.

The positive significant regression between the bile TPAH and the bile cPAH is due to the fact that six cPAH compounds are included in the 16 TPAH compounds (16), thus the level of TPAH compounds are expected to correlate positively with the cPAH compounds. Another reason for this positive correlation is that the metabolic products of the TPAH compounds will most often result in cPAH compounds with a conserved backbone structure that will preserve its affinity to the antibody combining site included in the enzymatic assay system. It is worth noting that such established correlations were most likely caused by the oil spill, seen by unaided eyes of the divers and fishermen as huge black sediments at the harvesting time of the fish.

It is worth noting that in the future, similar investigations should attempt to standardise the metabolite parameters measured by correlating them to a digestive status-parameter, such as the bile protein or biliverdin levels. In addition, the levels obtained of the measured parameters could have been caused by local inputs and might not have been a direct result of the oil spill. The local inputs are mainly raw sewage outlets in the Mediterranean Sea which contain used car engine oils from the petrol stations present across the shore of the east Mediterranean Sea.

In the future, the different sources of the parameters measured should be determined to help define new strategies that are designed to reduce contamination in the Mediterranean Sea.

Conclusion

This study indicates the possibility of substituting muscle with bile in the analysis in *Mugil* spp. to quantitate TPAH and TPCB, thus avoiding the lengthy procedure of grinding and lyophilisation wet muscle to transform the samples to a dry state. In addition, the bile cPAH was found to be predictable from the bile TPAH in *Mugil* spp. In the future, this could enable the estimation of the cPAH values from the determined TPAH using established regression relationships.

Acknowledgments

We are grateful to the Green Peace divers and other crew members on the *Rainbow Warrior* for identifying the six sites and for their determination of the coordinates using the GPS.

Grant support

The Task Force of the American University of Beirut kindly provided the funds required to accomplish this research.

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