# FATTY ACID PROFILE OF NEOGASTROPOD SPECIES *BABYLONIA SPIRATA* FROM MANORA CHANNEL, THE INTENSIVE SHIPPING AREA OF PAKISTAN

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## ABSTRACT

This work presents the fatty acids methyl esters (FAME) profile of neogastropod species *Babylonia spirata* from Manora Channel, the area subjected to intensive shipping activity. A total of 39 compounds including fatty acids methyl esters (FAME), dimethyl acetals (DMA) of fatty aldehydes, non-methylene-interrupted (NMI) acids were separated and identified in digestive gland/ gonad complex of males, morphologically normal females and imposex females (imposition of male sex characters onto females). Variability in components of saturated, monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids profiles was observed in males, females and imposex females. Increasing levels of MUFA fatty acids were observed in imposex females. The effect of organotin contamination on the accumulation and alteration of lipids and FAME composition has not been extensively investigated. This is the first report of FAME analysis of this species from Pakistan.

**Key-words**: Endocrine; Fatty acids; Neogastropods; Organotin; Pakistan

## INTRODUCTION

Uptake of famous organotin compounds; tributyltin and triphenyltin (TBT/ TPhT) by marine organism is known to disrupt endocrine system which results in masculanization by upsetting the hormone level in female gastropods known as imposex. It has been suggested and recommended that marine invertebrates, particularly prosobranch snails are most sensitive organisms to the effect of these endocrine disruptors (Bryan *et al*., 1991; Mathissen and Gibbs, 1988; Daft *et al*., 2001; Janer *et al*., 2007, Afsar *et al*., 2012c).

TBT is known to act on neurohormones that are responsible for controlling sexual differentiation in mollusks (Feral and LeGall, 1983). A number of studies have been carried out to investigate the effects of organotin compounds on sex steroid levels and metabolism in mollusks (Betin *et al*., 1996; Marcillo *et al*., 1998; Oberdoster *et al.*, 1988; LeBlance *et al*., 1999 and 2005; Gooding *et al*., 2003). Increased testosterone production and accumulation is an endocrine dysfunction and is responsible for imposex. High concentrations of TBT effects sex steroid hormones (testosterone and 17β-estradiol) thus result in the increase of free testosterone (unesterified) levels in imposex females, otherwise, very high concentration of TBT inhibits the testosterone-fatty acid esterification in imposex females (Oberdoster *et al*., 1998; LeBlance *et al*., 1999; Gooding *et al*., 2003; LeBlance *et al*., 2005).

The effect of TBT on the accumulation or alteration of lipids and their fatty acid methyl ester (FAME) in male, female and imposex females of gastropod species has not been extensively investigated. Janer *et al.* (2007)reported that exposure to TBT could affect lipid homeostasis in ramshore snail *Marisa cornuarietis*, when subjected to high concentrations of TBT. In this species 2-3 fold increase in lipid and fatty acids concentrations was observed in the digestive gland /gonad complex (Janer *et al.*, 2007).

In the present study fatty acid methyl ester (FAME) profiles were analyzed to examine the possible variability or alteration in FAME components, among male, females and imposex females of neogastropod species from Manora Channel the organotin contaminated site (Afsar *et al.*, 2010)being the hub of shipping and vessel related activities.

## MATERIALS AND METHODS

### Sample collection & preparation:

Specimens of neogastropod species, *B. spirata* were collected in June 2008 from the low tidal zone of Manora Channel. Soft tissue was extracted out by breaking the shell and the gonad and digestive gland complex of male, morphologically normal female and imposex female was dissected and wet mass of 2 g was kept in stoppered glass tubes containing 20-40 ml 2:1 v/v chilled chloroform/methanol (C:M) and were stored at -20 °C till further analysis. A total of three samples (1 male, 1 female and 1 imposex female) of *B.spirata* were prepared for FAME (fatty acid methyl ester) analysis. Each sample was comprised of pooled gland/gonad complex of four specimens. In case of imposex females the tissues of females with VDS (vas deferens sequence) stages 2 to 4+ were pooled as described by Afsar (2009).

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**Fatty acids Extraction:**

Extraction was based on the method described by Folch *et al*.(1957). Samples were homogenized by using Ultra Turrax TM and then 0.25 volumes of 0.88% (w/v) KCl added to homogenized samples. The homogenized samples were centrifuged at $400g_{ave}$ (1500 rpm Jouan C 412 bench centrifuge). The top layer was removed by aspiration and bottom layer was filtered with Whatman no. 1 filter paper. The solvent was evaporated to dryness under a stream of oxygen-free nitrogen (OFN) on a nitrogen evaporator and desiccated *in vacuo* overnight. Total lipids were re-dissolved in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoulene (BHT) as an antioxidant and stored at -20±5 ºC in a screw cap vial after gentle flush with nitrogen. Fatty acid methyl esters were prepared from total lipid by acid-catalyzed transestrification using 2 ml of methylating reagent (1%$H_2SO_4$ in Methanol). The tubes containing the samples were flushed with nitrogen and incubated overnight (16 hr) at 50 °C after addition of known amount of heptadecanoic acid (17:0) as internal standard. Finally fatty acid methyl esters (FAME) were extracted with iso-hexane: diethyl ether (1:1, v/ v). Methyl esters were purified by thin layer chromatography (TLC) on 20x20 cm plates (Silica gel G 60, Merck) using iso-hexane: diethyl ether: acetic acid (90: 10: 1) as the solvent system.

**GC and GC-MS analysis:**

FAMES were analyzed with a Gas-Chromatograph (Fisons MD800) equipped with a phenomenex ZB-WAX column (30 mt x 0.32mm x 0.25), and cold on-column injection system, using helium as carrier gas at a flow rate of 2.0 ml/ min. Initial oven temperature was kept at 50°C then raised to 225°C at a ramping temperature of 40°C/ min to 150°C then at 2°C/ min to 225°C and finally hold for 5 minutes at 225°C and 1ul of solution in iso-hexane was injected. Peaks were recorded and integrated on a personal computer using Chrom Card software (Fisons) and FAMES were identified by comparison with a known fish oil standard Marinol.

**RESULTS**

Gas-chromatography mass spectrometric technique was employed to analyze the lipid components and the possible variability or alteration of FAME composition in digestive gland/gonad complex in males, females and imposex females of *B. spirata.* A total of 39 compounds were detected with a number of carbons C14 to C24 in all sex categories. These consisted of 6 saturated, 10 monounsaturated fatty acids (MUFA), seven n-6 polyunsaturated (n-6 PUFA) and 8 n-3 polyunsaturated fatty acids (n-3 PUFA). Other 8 (PUFA) compounds consisted of polyenoic fatty acids (16: 2, 16: 3 and 16: 4), dimethyl acetals (DMA) of fatty aldehydes (18:0 DMA) and non-methylene-interrupted (NMI) acids; three non-methylene interrupted dienoic (20: 2, 22: 2 and 22:2 NMID) and a non-methylene interrupted trienoic (22: 3 NMIT) acids which contributed relatively in small proportions (Table 1).

Detailed description of fatty acid profile is given in Table 1 and Figure 1. Highest percentage of total saturated fatty acids was found in imposex females (35.65) followed by females (35.10%) and males (29.91%). Major saturated fatty acids were palmitic acid (16:0), stearic acid (18:0) and myristic acid (14: 0) Behanic acid (22:0) was not detected in females but was found in equal amount (0.15%) in imposex females and males.. Total MUFA constituted 28.14% in imposex females, 27.72% in females and 24.17% in males. In *B. spirata* 16:1n-7, 18:1n-9, 18:1n-7 and 20:1n-11 were the major MUFAs.

The higher concentration of total PUFA was found in males (45.92%). In females and imposex females it was 37.18% and 36.20% respectively. The highest percentage of total n-6 PUFA detected in males (9.83%) whereas, in imposex females it was 7.30% and in females amounted to 7.11%. In *B. spirata* 20: 4n-6 and 18: 2n-6 were the major n-6 PUFA. The highest percentage of total n-3 PUFA was observed in males which was 24.03% followed by 21.66% in females and 19.84% in imposex females. In all the samples examined 20: 5n-3, 22: 6n-3, 22: 5n-3 were the major n-3 PUFAs.

Other PUFAs comprised of 16: 2, 16: 3, 16: 4, 18: 0 DMA, 20: 2NMID, 22: 2NMID, 22: 3NMIT. They contributed 12.05% in males, 9.06% in imposex females and 8.40% in females (Table 1). In imposex specimens *B. spirata* the concentration C16 and C20 was slightly high compared to females. While lower concentrations of C14, C15, C18, C22 and C24 were recorded in imposex females compared to females (Table 2).

Table 1. Fatty acid composition (% total fatty acids) of total lipid from gonadal tissues of *Babylonia spirata.* M=Male; F=Female; IS= Imposex female.

| | M (n=4) | F (n=4) | IS (n=4) |
|---|---|---|---|
| 14:0 | 2.72 | 4.36 | 3.59 |
| 15:0 | 0.66 | 0.83 | 0.68 |
| 16:0 | 18.46 | 22.50 | 23.93 |
| 18:0 | 7.39 | 7.02 | 6.98 |
| 20:0 | 0.53 | 0.38 | 0.32 |
| 22:0 | 0.15 | 0.00 | 0.15 |
| 16:1n-9 | 0.16 | 0.29 | 0.19 |
| 16:1n-7 | 6.71 | 10.40 | 10.20 |
| 18:1n-9 | 4.39 | 6.59 | 5.63 |
| 18:1n-7 | 4.42 | 4.61 | 4.85 |
| 20:1n-11 | 4.31 | 2.55 | 3.53 |
| 20:1n-9 | 1.37 | 1.13 | 1.04 |
| 20:1n-7 | 2.03 | 1.62 | 2.19 |
| 22:1n-11 | 0.38 | 0.26 | 0.30 |
| 22:1n-9 | 0.16 | 0.00 | 0.11 |
| 24:1n-9 | 0.24 | 0.27 | 0.12 |
| 18:2n-6 | 1.59 | 1.03 | 1.28 |
| 18:3n-6 | 0.25 | 0.44 | 0.24 |
| 20:2n-6 | 1.37 | 0.98 | 0.77 |
| 20:3n-6 | 0.46 | 0.32 | 0.26 |
| 20:4n-6 | 3.95 | 2.58 | 3.04 |
| 22:4n-6 | 1.35 | 1.02 | 1.13 |
| 22:5n-6 | 0.86 | 0.75 | 0.59 |
| 18:3n-3 | 0.52 | 0.49 | 0.47 |
| 18:4n-3 | 0.51 | 1.01 | 0.88 |
| 20:3n-3 | 0.12 | 0.00 | 0.07 |
| 20:4n-3 | 0.33 | 0.68 | 0.43 |
| 20:5n-3 | 9.07 | 8.27 | 8.02 |
| 22:4n-3 | 0.00 | 0.00 | 0.00 |
| 22:5n-3 | 6.48 | 4.67 | 4.95 |
| 22:6n-3 | 7.00 | 6.55 | 5.01 |
| 16:2 | 0.57 | 1.10 | 1.02 |
| 16:3 | 0.30 | 0.84 | 0.69 |
| 16:4 | 4.42 | 0.28 | 0.19 |
| 18:0 DMA | 0.14 | 2.29 | 2.81 |
| 20:2NMID | 0.62 | 0.35 | 0.54 |
| 22:2NMID | 2.33 | 1.50 | 1.96 |
| 22:2NMID | 2.94 | 1.40 | 1.78 |
| 22:3NMIT | 0.75 | 0.65 | 0.07 |
| Total saturated | 29.91 | 35.10 | 35.65 |
| Total monounsaturated | 24.17 | 27.72 | 28.14 |
| Total n-6 PUFA | 9.83 | 7.11 | 7.30 |
| Total n-3 PUFA | 24.03 | 21.66 | 19.84 |
| Total other PUFA | 12.05 | 8.40 | 9.06 |
| Total PUFA | 45.92 | 37.18 | 36.20 |

Table 2.-*Babylonia spirata:* Levels of fatty acids by carbon chain length in gonadal tissues. M- males; F- females; IS- imposex females.

| Gender | ∑C14 | ∑C15 | ∑C16 | ∑C18 | ∑C20 | ∑C22 | ∑C24 |
|---|---|---|---|---|---|---|---|
| M | 2.72 | 0.66 | 30.61 | 19.21 | 24.15 | 22.41 | 0.24 |
| F | 4.36 | 0.83 | 35.42 | 23.47 | 18.85 | 16.79 | 0.27 |
| IS | 3.59 | 0.68 | 36.22 | 23.12 | 20.21 | 16.06 | 0.12 |

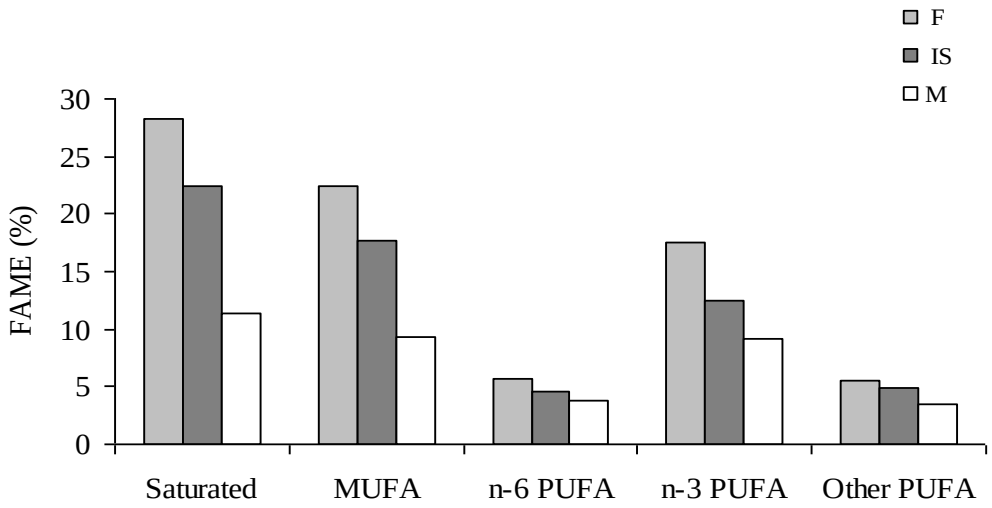


Fig. 1. Fatty acid composition (% total of dry weight) of males (M) females (F) and imposex females (IS) of *Babylonia spirata* at Manora channel. MUFA- monounsaturated fatty acids; PUFA- Poly unsaturated fatty acids).

## DISCUSSION

In the present study variability in components of fatty acid profiles of males, females and imposex females was evident. This is considered to be associated with TBT toxicity (Masia *et al.,* 1998; Janer *et al.,* 2007). FAME profile studies of *B. spirata* showed slightly higher concentration of monoenoic acid in imposex females were observed as compare to females. This may be to the fact that in Pakistan advance stages of imposex were not found in any species of gastropod (Afsar, 2009; Afsar *et al.,* 2010) because of the low intensity of boating activity compared to elsewhere in the world where studies on TBT toxicity have been carried out (Janer *et al.,* 2007). They have reported sharp increase in monoenoic fatty acids in specimens of *M. cornuarietis* subjected to high concentration of TBT (500ng as Sn/L), whereas, at low TBT concentration the difference was not so pronounced. Relative decrease in percentages of total PUFA was observed in imposex females. From Pakistan in the imposex related study *B. spirata* was found to be more sensitive to TBT toxicity as more advance stages of imposex and ovarian spermatogenesis was observed in this species (Afsar, 2009) and thus accumulated more TBT in the gonad/digestive gland complex. In specimens of *M. cornuarietis* treated with different concentrations of TBT, relative decrease of percentages of PUFA in female was found when exposed to high concentration of TBT (500 ng as Sn/ $l^{-1}$) whereas, in specimens treated with nominal concentration of TBT, 30 and 125 ng as Sn/ $l^{-1}$ there was a relative increase of PUFA in the gonad/digestive gland complex. Similar response was observed in males but the magnitude was not significant (Janer *et al.,* 2007).

In addition to this comparative studies of fatty acids in marine, brackish and freshwater animals reveals that the diet, physiological status and environmental factors could also influence fatty acid composition in invertebrates and fish (Dembitsky *et al.,* 1993b, Dembitsky and Razanka 1996; Mustafa and Nakagawa, 1995; Paradis and Ackman, 1977; Ackman 2000; Go *et al.,* 2002). Janer *et al*. (2007) reported in ramshorn snail *M. cornuarietis* that TBT also alter fatty acid profile. The overall change was observed in terms of degree of unsaturation and carbon chain length. The total concentration of C18 fatty acids was increased whereas, the concentration of C14, C15, C17, C20, C21 and C22 decreased in both male and females exposed to TBT. The imposex females of *B. spirata* have shown increasing trend in concentration of C16 and C20. The concentration of C14, C15, C18, C22 and C24 decreased in females. In gonad/digestive gland complex of ramshorn snail, *M. cornuarietis* at high levels of TBT two fold increase in the percentage of total lipids and 2.7 fold increase in fatty acids levels was recorded in females but no significant effect in exposed males was observed. Only a slight shift in fatty acid profile at different concentrations of TBT was encountered in this species. Increase in the level of total lipid has been considered parallel to the decrease of arachidonic, eicosapentaenoic, docosahexaenoic and other PUFAs, because PUFAs are known to inhibit lipogenesis (Janer *et al.,* 2007). In addition to this PUFAs are also considered as signaling molecules that act through signaling pathway PPARs (peroxisome-proliferator activated receptor) which can promote or inhibit adipogenesis (mass production of new fat cells) in mammals (Madsen *et al.,* 2005).

Fatty acid methyl esters (FAME) profile analysis of *B. spirata* was carried out on the specimens procured from Manora Channel. The study revealed the alteration in some components of FAME profiles in male, female and imposex females of the species which is due to possible organotin contamination effects on endocrine system of the native candidate species. This is the first report of the fatty acid profiles of *B. spirata* from Pakistan.

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