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Virgin microplastics cause toxicity and modulate the impacts of phenanthrene on biomarker responses in African catfish (*Clarias gariepinus*)

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ABSTRACT

Despite the ubiquity of microplastics (MPs) in aquatic environments and their proven ability to carry a wide variety of chemicals, very little is known about the impacts of virgin or contaminant-loaded MPs on organisms. The primary aim of this study was to investigate the impacts of virgin or phenanthrene (Phe)-loaded low-density polyethylene (LDPE) fragments on a suite of biomarker responses in juvenile African catfish (*Clarias gariepinus*). Virgin LDPE (50 or 500 μg/L) were preloaded with one of two nominal Phe concentrations (10 or 100 μg/L) and were exposed to the fish for 96 h. Our findings showed one or both Phe treatments significantly increased the degree of tissue change (DTC) in the liver while decreased the transcription levels of forkhead box L2 (*foxl2*) and tryptophan hydroxylase2 (*tph2*) in the brain of *C. gariepinus*. Exposure to either levels of virgin MPs increased the DTC in the liver and plasma albumin: globulin ratio while decreased the transcription levels of *tph2*. Moreover, MPs modulated (interacted with) the impact of Phe on the DTC in the gill, plasma concentrations of cholesterol, high-density lipoprotein (HDL), total protein (TP), albumin, and globulin, and the transcription levels of *fushi* tarazu-factor 1 (*ftz-f1*), gonadotropin-releasing hormone (*GnRH*), 11 *β*-hydroxysteroid dehydrogenase type 2 (*11β-hsd2*), and liver glycogen stores. Results of this study highlight the ability of virgin LDPE fragments to cause toxicity and to modulate the adverse impacts of Phe in *C. gariepinus*. Due to the wide distribution of MPs and other classes of contaminants in aquatic environments, further studies are urgently needed to elucidate the toxicity of virgin or contaminant-loaded MPs on organisms.

1. Introduction

Since the mass production of plastics in the 1940 s, these polymers have become an indispensable part of modern life. Plastics are widely used by different industries due to their low cost, elasticity and durability, and it is estimated that 299 million tonnes are produced each year (PlasticsEurope, 2014). Autocatalytic, thermooxidative, photo-oxidative, and biological degradations are the processes involved in converting large plastic pieces (macroplastics) into smaller particles (Andrady, 2011; Gregory and Andrady, 2003) called microplastics, hereafter presented as MPs, which are defined here as being from 1 to 1000 μm in size. In aquatic environments, MPs are often found in several major physical forms: fibres and films (Lusher et al., 2013; Mathalon and Hill, 2014), fragments (von Moos et al., 2012), and beads (de Sá et al., 2015). Polyester and acrylic fibres are believed to be mainly derived from washing clothes (Rillig, 2012) while fragments are nonuniformly-shaped microparticles primarily produced via the fragmentation of macroplastics (Hidalgo-Ruz et al., 2012). Fragments were chosen for this study because they are among the most predominant MP forms found in aquatic environments (do Sul et al., 2013) and in fish (Boerger et al., 2010; Lusher et al., 2013).

High-density polyethylene (HDPE) and low-density polyethylene (LDPE) correspond to 12% and 17% of the total European plastics used, respectively (PlasticsEurope, 2010). They have been

Abbreviations: MPs, Microplastics; PE, Polyethylene; LDPE, Low-density polyethylene; Phe, Phenanthrene; *GnRH*, Gonadotropin-releasing hormone; *tph2*, Tryptophan hydroxylase2; *cyp19a2*, Brain aromatase; *foxl2*, Forkhead box L2; *ftz-f1*, *Fushi* tarazu-factor 1; *11β-hsd2*, 11 *β*-hydroxysteroid dehydrogenase type 2; TP, Total protein; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; LDH, Lactate dehydrogenase; ALP, Alkaline phosphatase; γGT, Gamma-glutamyl transpeptidase; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; DTC, Degree of tissue change

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reported as the predominant polymer type in freshwater ecosystems (e.g., Corcoran et al., 2015). Because of their positive buoyancy, surface currents and the sun's ultraviolet light facilitate their fragmentation, leading to increased secondary sources of polyethylene (PE) MPs (Cole et al., 2011).

Over the past few years, the number of studies on MPs is rapidly increasing. Most of these studies, however, have focused on the occurrence and distribution of MPs in the environment (e.g., Frias et al., 2016) and across a range of feeding guilds (Desforges et al., 2015; Neves et al., 2015). Biomarker responses have been intensively used to evaluate the impacts of stressors on fish (see review by Van der Oost et al., 2003). In European sea bass (*Dicentrarchus labrax*) larvae, PE microspheres did not affect the growth rate or Interleukins-1-beta (*IL-1b*) transcription level, but increased mortality rates at 14 and 20 days post-hatch, possibly due to blockage of the intestinal lumen (Mazurais et al., 2015). Given the diversity of contaminants in aquatic environments, biomarker responses in fish are the net result of chemical interactions (Rudneva, 2013). In aquatic environments, MPs can adsorb various contaminants including polycyclic aromatic hydrocarbons (PAHs, Teuten et al., 2007), which are widely distributed in marine and freshwater ecosystems (Boonyatumanond et al., 2006; Keshavarzifard et al., 2015). Among them, phenanthrene (Phe) is a widespread PAH in contaminated sites (Maltby et al., 1995; Wang et al., 2009) and have been shown to cause toxicity in fish (Karami et al., 2008; Sun et al., 2006) and humans (Hecht et al., 2010). Oliveira et al. (2013) showed that the addition of PE microspheres to the pyrene-spiked test solutions delayed pyrene-induced death to the common goby (*Pomatoschistus microps*) juveniles, increased the concentration of biliary pyrene metabolites, and reduced isocitrate dehydrogenase (IDH) activities.

The selected Phe concentrations that were used in the current study were below the reported median lethal concentration (LC_{50}) values for different fish species (150–1410 μg/L; Lee et al., 1986; Sogbanmu and Otitoloju, 2013; Zhao et al., 2011). In addition, the chosen concentrations are within the range that have been shown to cause significant changes in biomarker responses in fish (Jee and Kang, 2005; Sun et al., 2006). No information is available on the concentrations of MPs smaller than 100 μm in freshwater ecosystems. The MP concentrations used in the current study were within the range reported for larger MPs or macroplastic particles in the freshwater bodies (e.g., Moore et al., 2011), and also the concentrations used by the earlier toxicity testing (Davarpanah and Guilhermino, 2015; Oliveira et al., 2013).

African catfish (*Clarias gariepinus*) is becoming one of the most widely accepted model fish species in toxicological studies (Karami et al., 2015; Karami and Courtenay, 2015). To investigate the impact of virgin or Phe-loaded MPs on *C. gariepinus*, a suite of molecular, biochemical, and histopathological biomarkers was employed. Two null hypotheses (H_0) were examined in this study: (H_{01}) exposure to virgin MPs does not affect biomarker responses in *C. gariepinus*, and (H_{02}) MPs do not modulate (interact with) the impacts of Phe on biomarker responses in *C. gariepinus*.

There are several concerns pertaining to the available literature on the impacts of MPs on aquatic organisms such as employing polymers of precise sizes and homogeneous shape (i.e. microbeads) (Phuong et al., 2016). Furthermore, limited studies have presented the chemical analysis of tested MPs (e.g., Rochman et al., 2013) or in mixed exposure experiments, with only few studies pre-incubating MPs with the target contaminant (e.g., Batel et al., 2016). To address these concerns, this study developed a stepwise bioassay protocol to investigate the toxicity of concurrent exposure to virgin or contaminant-loaded MPs in fish.

2. Materials and methods

A flow diagram of the experimental design is presented in Appendix A.

2.1. MPs characterization

The model MPs were virgin LDPE powder with nonuniformly shaped fragments (with $>95\%$ of particles were <60 μm in size) supplied by Toxemerge Pty Ltd (Australia).

2.1.1. Particle size, count, and morphology

Particle size distributions of the MPs were determined in triplicate by laser diffraction using *Malvern* Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK). Morphology of MPs were characterised by observing the platinum-coated fragments under a field emission scanning electron microscopy (FESEM, JOEL 7600 F). Finally, fragments were counted using a hemocytometer.

2.1.2. Raman spectroscopy

Raman spectra of three random samples were recorded using a Raman spectrometer equipped with a grating (600 grooves mm^{-1}) spectrometer (WITec, UHTS 300) and a charge-coupled device (CCD) detector (Andor, DV401-BV). Then, the spectra were compared against a reference database (KnowItAll ® Informatics System).

2.2. Preparation of Phe-loaded MPs

In this study, MPs belonged to two groups: virgin MPs (used in the MP treatments) and Phe-loaded MPs (used in the MP + Phe treatments). For the exposure to Phe-loaded MPs, we did not add MPs directly to the Phe-spiked test tanks due to inconsistencies in the concentration of adsorbed Phe among different MPs. Instead, fragments were preloaded with one of the two Phe concentrations in the water 28 h before the exposure to ensure the MPs were saturated with Phe.

A two-phase preparation protocol was performed to ensure similar Phe concentrations across all MP fragments. Phase I was executed to determine the measured Phe concentrations after a simulated exposure experiment when nominal concentrations of 10 and 100 μg/L was used. After determining the measured concentrations, which were 4.12 and 35.5 μg/L, these were then used in Phase II to preload the MPs under conditions that would prevent degradation / absorption of Phe. This would then ensure the marginal differences between the nominal and measured concentrations (4.12 and 35.5 μg/L). Having similar Phe concentrations across Phases I and II, there would be minimal uptake of Phe from the water onto the Phe-loaded MPs or the loss of Phe from the Phe-loaded MPs into the water during the exposure experiment. This step-wise procedure would minimize the risk of committing type I or II errors.

The duration necessary for MPs to sorb chemicals at an equilibrium state from the surrounding environment varies mainly according to the chemical composition of the plastic (Fries and Zarfl, 2012; Teuten et al., 2007). By using a Micromeritics Gemini 2375 Surface Area Analyzer, Teuten et al. (2007) found that PE has the largest surface area among several other tested polymers. Also, they reported that PE possesses the highest equilibrium distribution coefficient (Kd) for Phe. In an aqueous phase, within 7.8–8.3 h, LDPE fragments reach an equilibrium state (t_{90}) with Phe (Fries and Zarfl, 2012). A longer duration of 28 h was used to ensure homogeneous Phe loading on all the fragments.

2.2.1. Phase I

In our earlier study we showed a considerable difference

between the nominal and measured Phe concentrations (Karami et al., 2016). Several factors are responsible for the observed lower measured than nominal concentrations such as biological degradation, photooxidation, and absorption to silicone sealants. In the case of the latter, this was used to construct our experimental aquaria and has a demonstrated strong tendency for absorbing hydrophobic substances (OECD, 1992). A fresh stock solution (5 mg/mL) of Phe (98% pure, Sigma–Aldrich, Germany) in HPLC–grade ethanol (Fisher Scientific) was prepared and added to six 324 L glass aquaria to obtain two nominal Phe concentrations (10 or 100 μg/L, with three aquaria per concentration). After 24 h, the test solutions were completely renewed, and juvenile *C. gariepinus* [mean (SD): 121.00 (13.02) g in weight and 26.90 (2.53) cm in length] were randomly distributed among the aquaria filled with 286 L of UV-treated dechlorinated tap water (one fish per aquarium). Phenanthrene concentrations in each treatment (10 and 100 μg/L) were quantified by sampling the water from each aquarium 30 min and 24 h after the addition of Phe. Phenanthrene residues were extracted using a liquid-liquid extraction method and detected by HPLC-UV as described earlier (Karami et al., 2016). The average of the measured Phe concentrations per treatment (3 aquaria $\times 2$ samplings $=6$) were used to prepare the Phe-sorbed MPs in Phase II.

2.2.2. Phase II

To prepare the Phe-sorbed MPs, a stock suspension of LDPE fragments (18 mg/mL) was prepared in HPLC–grade ethanol. Due to the positive buoyancy of LDPE (specific gravity 0.91–0.94 g/cm^3) and tendency for aggregation on the water surface, we used a less dense solvent (ethanol, specific gravity 0.79 g/cm^3) to prepare a homogeneous suspension. Working solutions of MPs (143 or 14.3 mg/L corresponding to final concentrations of 50 and 500 μg/L, respectively, in 286 L water in the exposure experiment) were prepared by adding the stock suspension to 1 L Schott bottles filled with UV-treated dechlorinated tap water. The test solutions were spiked with the Phe stock solution to reach final concentrations of 4.12 or 35.5 μg/L (these values had been obtained from Phase I). The Schott bottles were wrapped in aluminium foil, capped, and shaken at 200 rpm on a horizontal shaker under darkness for 28 h at room temperature. The solution was changed once after 14 h. Afterwards, the media was filtered through a cellulose acetate membrane (0.2 μm pore size, Whatman International Ltd.) and washed thrice with ultrapure water. These Phe-loaded MPs were used in the exposure experiment. This process had been initiated about 28 h prior to the start of the exposure experiment and repeated every day to produce fresh Phe-loaded MPs.

2.3. Chemical analysis of MPs

Virgin and Phe-loaded MPs were analyzed for PAHs, PCBs, heavy metals, and phthalates. All glassware were meticulously cleaned: washed with detergent and water, rinsed twice with ultra-pure distilled water, then rinsed with acetone and hexane and finally heated in an oven. Microplastic samples were weighed in grams up to four decimal places. In each assay, a blank test was carried out in parallel using the same quantities of all reagents. The recovery rates (%) of the analytical procedure were calculated from spiking virgin MPs at two concentration levels for each element.

All reagents and solvents were analytical or HPLC grade. Mixtures of PAHs (EPA M-610) and PCBs standards (purities $\geq 95\%$) were supplied by AccuStandard (New Haven, CT, USA). Phthalates standards were purchased from Sigma–Aldrich (Mississauga, Canada), and stock standard solutions (1000 mg/L) of arsenic, cadmium, and lead were from Sigma–Aldrich (Milwaukee, WI, USA).

2.3.1. Determination of heavy metals

Concentrations of arsenic, cadmium, and lead on MPs were determined according to EPA Method 6010B. A MP sample of one gram was added to HNO_3 and left for two hours. Afterwards, HCl was added and slowly heated on a hot plate to 80 °C for three hours. The reaction vessel was cooled to room temperature, and 10 mL of 6 N HCl was added. Serial dilutions of heavy metals (0.01, 0.05, 0.10, 0.50, 1.00 mg/L) were prepared by diluting the stock solutions. Extracts were analyzed for the heavy metals by an inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Perkin Elmer Optima 7500DV) according to the method of Chang et al. (2010). Efforts were made to quantify the concentration of mercury in MP samples using a cold vapour system (CVAAS), based on AOAC 971.21 (AOAC, 2002). However, due to low recovery rate ($<30\%$), this element was excluded from further analyses. The detection limits (LODs) were defined as three times the signal to noise (S/N) ratio and ranged from 1.8 to 4.00 ppb (part per billion) for the tested heavy metals.

2.3.2. Determination of PCBs and phthalates

Samples were extracted using an in-house method based on the method of Kozyrod and Ziaziaris (1989). Briefly, one gram of the MP sample was mixed with n-heptane, followed by the addition of $MgSO_4$: NaCl (5:1) to the mixture, vortexed for 30 min, and sonicated for 1 h. The mixture was centrifuged, and supernatant was used for the analyses. The extracts were analyzed for 15 PCBs (PCB1, PCB4, PCB18, PCB31, PCB81, PCB101, PCB110, PCB137, PCB153, PCB167, PCB169, PCB180, PCB183, PCB187, PCB206) and 6 phthalates [Bis(2-ethylhexyl) phthalate (DEHP), Di(*n*-octyl) phthalate (DNOP), Benzylbutyl phthalate (BBP), Diisodecyl phthalate (DIDP), Di-n-butyl phthalate (DBP), Diisononyl phthalate (DINP)] with a Hewlett-Packard (HP) 6890/5973 gas chromatography/mass spectrometry (GC/MS) equipped with a split/splitless injector, Agilent 7683B series autosampler, and a DB-5MS ™ (30 m $\times$ 0.25 mm id, 0.25 μm film thickness; J&W Scientific, Folsom, CA) capillary column. The GC/MS was calibrated with five different known concentrations of analytes. The LODs for individual PCBs and phthalates ranged from 0.10 to 2.20 ppb and 0.11-0.56 ppb, respectively.

2.3.3. Determination of PAHs

Microplastic samples were extracted for their contents of 16 PAHs [acenaphthene, acenaphthylene, benz[a]anthracene, benzo[g,h, i]perylene, benzo[k]fluoranthene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorine, indeno[1,2,3-cd]pyrene, 2-methylnaphthalene, naphthalene, Phe, pyrene, benzo[*b*]fluoranthene, and benzo[a]pyrene] based on an in-house method according to Zhao and Zhai (2010). A 5-g MP sample was added to ultra-pure water and shook vigorously for 1 min, followed by the addition of acetonitrile and shook for 30 s. The extraction process was continued by using an Agilent SampliQ QuEChERs EN extraction packet (4 g of $MgSO_4$, 1 g anhydrous NaCl, 1 g of sodium citrate and 0.5 g of disodium citrate sesquihydrate). The sample tubes were centrifuged at 4000 rpm for 5 min. The cleanup process was started by transferring 6 mL of supernatant into a 15-mL SampliQ QuEChERs EN Dispersive SPE tube containing 150 mg primary-secondary amine (PSA), 150 mg C18 and 900 mg of anhydrous $MgSO_4$) and continued by shaking and centrifugation at 4000 rpm for 5 min. A 4-mL aliquot of extract sample was filtered through a 0.22 μm filter membrane. Sample extracts were analyzed using an HP 6890/5973 GC/MS as described above. The LODs for individual PAHs ranged from 0.2 to 4.90 ppb.

2.4. Experimental design

2.4.1. Fish breeding

Broodstocks were reared in 2000 L fiberglass tanks for 12 months. Fish were bred by the injection of Ovaprim® (Karami et al., 2011) and fertilized eggs were transferred to fiberglass tanks filled with UV-treated dechlorinated tap water. Fish were initially fed *ad libitum* on commercial powder (Cargill, crude protein: 38–40%) and then on pellets (Star Feed, crude protein: Min 45%) for 11 weeks until they were used in the experiment.

2.4.2. Exposure experiment

A total of fifty full-sibling juvenile *C. gariepinus* were randomly distributed among 324 L aquaria (one fish per aquarium, five aquaria per treatment) one week before the start of the experiment and fed twice a day at 5% body weight. The study was approved by the Animal Ethics Committee of Universiti Putra Malaysia (reference no: UPM/IACUC/AUP- R093/2014). Exposure were executed according to OECD guidelines (OECD, 1992) with some changes. The experiment was comprised of the following treatments: negative control (NC), solvent control (SC; ethanol: $<0.01\%$); MP treatments (50 μg/L MPs, 500 μg/L MPs), Phe treatments (10 μg/L Phe, 100 μg/L Phe), and Phe-loaded MP treatments (50 μg/L MPs + 10 μg/L Phe, 50 μg/L MPs + 100 μg/L Phe, 500 μg/L MPs + 10 μg/L Phe, and 500 μg/L MPs + 100 μg/L Phe). The Phe-loaded MPs produced in Phase II were used in the MP + Phe treatments. In the MP + Phe treatments, the test solutions were first spiked with a sufficient volume of the stock Phe solution (5 mg/mL) and then the corresponding amounts of Phe-loaded MPs (produced by Phase II) were added to the aquaria.

The experiment was executed in a 12:12 light-dark cycle. The mean values (SD) for water quality were (n=20): temperature 27.7 (0.9)°C, pH 6.42 (0.49), dissolved oxygen 6.71 (0.53) mg/L, alkalinity 43.7 (4.07) mg $CaCo_3$, hardness 59.8 (6) mg $CaCo_3$, and salinity <1 mg/L. Aquaria were aerated with an airstone connected to a central blower. In each aquarium, a submersible water pump (JI YIN 807, China) was installed just below the water surface to minimize the aggregation of MPs. The pumps had been incubated in their corresponding Phe solutions for 48 h prior to the start of the experiment. Throughout the 96 h exposure period, fish were fed twice a day at 2% of body weight: in the morning with floating commercial pellets sprinkled on the water surface (Star Feed, crude protein: min 32%) and in the afternoon with commercial sinking pellets (Star Feed, crude protein: min 40%). Test media were changed every 24 h with UV-treated and dechlorinated tap water spiked with appropriate Phe / MP concentrations. Feeding was ceased 18 h before the end of the exposure period. After a 96-h exposure, fish were euthanized with an overdose of clove oil (Karami et al., 2012). Immediately, blood samples were taken and transferred into ethylenediaminetetraacetic acid (EDTA)-treated Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 3000 rpm for 15 min at 4 °C. Whole brains were sampled, snap-frozen in liquid nitrogen, and stored at −80 °C until RNA isolation two months later. The gill and liver tissues (n=4) were quickly removed and fixed in 10% (v/v) phosphate-buffered formalin.

2.4.3. Confirmation of MPs consumption

To confirm whether MPs were taken up by individual fish, intestinal materials were aseptically removed, incubated in 10% potassium hydroxide solution at 40 °C for 48 h, and finally filtered on a glass microfiber filter GF/F 0.7 μm (Whatman). The recovered MPs were viewed under an Olympus polarizing microscope (BX50, Olympus, Japan).

2.4.4. Phe concentrations in the test solutions

Samples of water (500 mL) were collected from all the treatments in amber coloured Schott bottles 30 min before and after the daily water changes, and filtered on a glass microfiber filter. Phenanthrene residues were extracted and quantified as explained in details earlier (Karami et al., 2016).

2.5. Biomarkers determination

2.5.1. Histology and liver glycogen store

Histopathological slides of the liver and gill tissues were obtained according to standard methods. Briefly, formalin-fixed tissues were dehydrated in graded concentrations of ethanol, cleared in xylene and embedded in paraffin wax. Several paraffin wax sections (5 μm) per tissue were obtained and then stained with hematoxylin-eosin (H&E). Pictures were taken with a light microscope (Leica DM750, Imager ICC50; 20× and 40×). Degree of tissue change (DTC) was calculated by classifying pathological lesions into three stages (Appendix C) according to a slightly modified methods of Poleksic and Mitrovic-Tutundzic (1994): Stage I represented normal tissue, Stage II indicated changes that would disrupt normal function of the associated tissue, and Stage III were changes that were considered severe and irreparable. The DTC was then calculated by counting the presence of each stage from 25 random secondary lamellae or liver pictures equally obtained from each of the four replicate tissues per treatment. The percentages of stage I, II and III from each replicate were then multiplied by 1, 10 or 100, respectively, to yield the DTC value. All sections were viewed under a polarizing microscope to investigate the presence of MPs within the tissues.

To investigate glycogen stores, the liver tissue sections were stained with Periodic acid-Schiff (PAS) stain in one session. Control sections were pre-treated in 0.5% α-amylase (Sigma Aldrich) for 20 min at 37 °C to digest glycogen and then stained with PAS stain (Sheeham and Hrapchak, 1987). Staining area values were produced for 12 randomly taken images from each of the 4 replicate livers per treatment using ImageJ software employing the same lower and upper threshold (0 and 145, respectively) for each picture.

2.5.2. Plasma biochemical determinations

Lipase, total protein (TP), albumin, total and direct bilirubin, glucose, lactate, aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (γGT), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, and cholesterol were measured in duplicate on a Hitachi 902 automatic clinical analyzer (Boehringer, Mannheim, Germany) using standard diagnostic kits (Roche Diagnostic GmbH, Mannheim, Germany). Globulin concentrations were calculated by subtracting TP from the albumin concentration. Our within-run precision coefficients of variance ranged between 3.5% for ALT and 6.7% for HDL.

2.5.3. Gene transcription levels

The RNA content of the brain samples were extracted with a RNeasy Mini kit (Qiagen, Valencia, CA) and reverse-transcripted by the use of a Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Gene-specific oligonucleotide primers (Appendix B) were purchased from First Base (Singapore). Gene transcription level was performed by quantitative reverse-transcriptase PCR (qRT-PCR; Bio-Rad CFX Connect real time system, Hercules, CA, USA) using QuantiNova SYBR Green PCR kit (Qiagen, Valencia, CA). Changes in gene transcription levels were normalized against the transcription of *GAPDH* and *β-actin* (housekeeping genes). These genes have commonly been used as internal standards for quantitative RNA

analysis (e.g., Chu et al., 2011) and their expressions were relatively stable throughout the current study [coefficient of variation (Ct CV%) < 3%]. All samples were assessed in duplicate. Changes in gene transcriptions were evaluated with the $2^{-\Delta\Delta CT}$ calculation method.

2.6. Data analysis

Before the analyses, all data had been checked for normality (Shapiro-Wilks test) and homoscedasticity (Levene's test). Data were log transformed when necessary and appropriate. For each parameter, NC and SC groups were compared by Student's *t*-test or a one-way permutational multivariate analysis of variance (one-way PERMANOVA, Anderson, 2001). Negative and solvent control groups were pooled if no differences were found. Otherwise, NC was dropped if the difference was significant (OECD, 2011). Data of blood biochemical parameters, glycogen contents, and gene transcription were analyzed by two-way ANOVAs. If ANOVA showed a significant difference ($P < 0.05$), the analysis was followed by Duncan's multiple range test. The strength of relationships between lipids and proteins were assessed using Pearson correlations. Parametric tests were analyzed with IBM SPSS Statistics (V. 22). Degree of tissue changes in the liver and gill were tested with two-way PERMANOVAs employing Euclidean distances and 9999 permutations (Anderson, 2001) using the PAST® software. To minimize the chances of committing type II errors, due to running multiple tests, significant main factors or interactions were further investigated by pairwise comparisons using a Holm–Bonferroni adjusted P value (Holm, 1979).

3. Results

3.1. LDPE fragment characteristics

Fig. 1 shows a FESEM image of LDPE fragments. Appendix D presents the mean (SD) volume (%) versus the MP size range (μm). Raman spectrum of the LDPE is shown in Appendix E. According to the haemocytometer counting analyses, the particle count in the 50 and 500 μg/L MP treatments were equivalent to about 1400 and 14,000 particles/L, respectively.

3.2. Concentration of Phe in the water samples

The measured Phe concentrations in the water samples from different treatments are shown in Table 1. Changes in Phe concentrations before and after water changes across different treatments are given in Appendix F. Within each Phe concentration (10 or 100 μg/L), no differences in measured Phe concentration between the Phe and Phe + MP treatments were observed (Student's *t*-tests, $P > 0.05$).

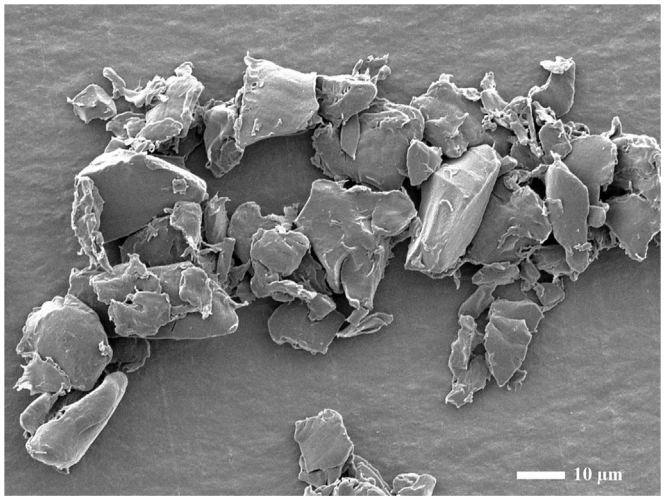

Fig. 1. Field Emission scanning electron microscope (FESEM) image of virgin LDPE fragments used by this study.

Table 1
Measured phenanthrene concentrations in the water samples of different treatments. Data are mean (SD), n=8.

Treatment		Measured phenanthrene concentration (μg/L)
Phenanthrene (μg/L)	Microplastics (μg/L)	
10	0	3.76 (1.18)
10	50	3.10 (1.45)
10	500	4.48 (1.99)
100	0	37.9 (4.11)
100	50	32.7 (5.85)
100	500	40.7 (4.35)
0	50	ND*
0	500	ND
Negative control		ND
Solvent control		ND

* ND: Not detected (below the limit of detection).

3.3. Concentrations of chemicals on MPs

Recoveries of the spiked MPs ranged from 75 to 102% for the tested heavy metals, for PCBs these were 68–100%, 72–104% for phthalates, and 75–105% for the PAHs. Loads of PAHs, PCBs, heavy metals, and phthalates on the virgin MPs were below the LODs. In Phe-loaded MPs, PCBs, heavy metals, and phthalates loads were below the detection limit. Except for Phe, the concentrations of other PAHs on the Phe-loaded MPs were also below the detection limit. The concentrations of Phe on the MPs incubated in the 4.12 or 35.5 μg/L Phe-spiked solutions were 0.5 and 41.1 mg/kg, respectively.

3.4. Biomarker responses

Table 2 presents a two-way ANOVA table on the effects of Phe or MPs, and their interactions on plasma biochemical parameters and brain transcription levels.

3.4.1. Degree of tissue changes and glycogen stores

There were no significant differences in DTC of the gill and liver between NC and SC groups (one-way PERMANOVA, $P > 0.05$). The gills in the NC and SC showed normal structure with numerous basal and mucous cells in the secondary lamellae and visible red blood cells in the capillary lumen. Similarly, the liver of catfish in the control and solvent treatments showed normal sinusoid structure and hepatocytes with uncongested blood vessels and occasional vacuoles.

The interaction term between MPs and Phe was significant for the DTC in the gill (two-way PERMANOVA, pseudo-F=26.94, $P < 0.05$, Fig. 2a) but not liver (two-way PERMANOVA, $P > 0.05$). Fig. 3a–e shows the histological changes in the gill tissues across some of the treatments. The gills of the fish exposed to 50 μg/L MPs showed some basal cell hyperplasia and sloughing as well as necrosis in the connective tissue (Fig. 3b), however when the MP concentration was increased to 500 μg/L, more severe changes were observed. This included epithelial lifting, hyperplasia, necrosis of the connective tissue, extensive cell sloughing, desquamation leading to the blood vessel exposure, and in some instances, a total loss of the secondary lamellae (Fig. 3c). In the case

Table 2
Two-way ANOVA table on the effects of Phe or MPs, and their interactions on plasma biochemical parameters and brain gene expressions. df=2 for the main factors and df=4 for the interaction terms; df error=41 for all biomarker responses except for glycogen where df=40.

Source of variation	Biomarker	F[1]	P[2]
Phenanthrene	Cholesterol	1.02	0.37
	HDL	3.68	< 0.05*
	Total protein	4.80	< 0.05*
	Albumin	3.75	< 0.05*
	Globulin	3.49	< 0.05*
	Albumin: Globulin	0.86	0.43
	ftz-f1	4.78	< 0.05*
	foxl2	6.73	< 0.05*
	GnRH	8.54	< 0.05*
	tph2	5.93	< 0.05*
	11β-hsd2	0.07	0.93
	Glycogen	55.3	< 0.05*
Microplastics	Cholesterol	0.74	0.48
	HDL	0.66	0.52
	Total protein	4.96	< 0.05*
	Albumin	4.20	< 0.05*
	Globulin	6.14	< 0.05*
	Albumin: Globulin	7.35	< 0.05*
	ftz-f1	3.72	< 0.05*
	foxl2	1.37	0.26
	GnRH	9.74	< 0.05*
	tph2	7.13	< 0.05*
	11β-hsd2	1.32	0.28
	Glycogen	58.2	< 0.05*
Phenanthrene × Microplastics	Cholesterol	10.1	< 0.05*
	HDL	5.81	< 0.05*
	Total protein	4.62	< 0.05*
	Albumin	3.58	< 0.05*
	Globulin	3.59	< 0.05*
	Albumin: Globulin	2.06	0.11
	ftz-f1	3.13	< 0.05*
	foxl2	1.16	0.34
	GnRH	5.09	< 0.05*
	tph2	2.34	0.07
	11β-hsd2	3.65	< 0.05*
	Glycogen	54.5	< 0.05*

* Represents significant difference at $P < 0.05$.
[1] F value (F statistic).
[2] P-value.

of the latter finding, this only occurred in the Phe-loaded MP treatments (e.g., Fig. 3e) and not in the 10 or 100 μg/L Phe treatments without MPs (Fig. 3d). Severe hyperplasia was also observed in all treatments with Phe that sometimes led to the total fusion of the secondary lamellae, but this was not observed in the virgin MP treatments (Fig. 3b and c).

Fig. 4a–e shows the histological changes in the liver across some of the treatments. The interaction term (MPs × Phe) of the liver DTC was not significant ($P > 0.05$). However, exposure to 500 μg/L MPs significantly affected DTC in the liver (two-way PERMANOVA, pseudo-F=13.03, $P < 0.05$; Fig. 2b). Similarly, exposure to either levels of Phe significantly increased liver DTC (two-way PERMANOVA, pseudo-F=46.89, $P < 0.05$; Fig. 2c). Some of the changes to the liver included necrosis, hemorrhaging and odemia. Meanwhile, the PAS staining intensity (glycogen store) across MP concentrations were significantly different among the Phe treatments (Fig. 5).

3.4.2. Blood biochemistry

Among the tested parameters, the only significant difference between SC and NC groups was observed for LDH activity (Student's t-test, $t(8)=2.72$, $P < 0.05$). Exposure to virgin or Phe-loaded MPs had no effect on plasma lipase, total and direct bilirubin, lactate, AST, ALT, ALP, γGT, LDL, and triglycerides. The lowest virgin MPs level (50 μg/L) or the highest Phe level (100 μg/L) significantly reduced the plasma cholesterol levels (Fig. 6a). In addition, both virgin MP treatments significantly lowered blood HDL levels (Fig. 6b). Similar to cholesterol and HDL, exposing the fish to 50 μg/L virgin MPs significantly decreased the plasma globulin level (Fig. 6d), while Phe-loaded MPs (500 μg/L MPs + 10 μg/L Phe) increased plasma globulin and TP (Figs. 6d and 6e). Exposure to 50 μg/L virgin or Phe-loaded MPs significantly increased the albumin: globulin ratio (Fig. 6f). Changes between lipids (i.e. cholesterol and HDL) and proteins (i.e. TP, albumin, and globulin) followed a similar pattern throughout the study (Pearson's correlation, $P < 0.05$, r value ranging from 0.55 between HDL and albumin to 0.90 between TP and globulin).

3.4.3. Gene transcription levels

There was a significant difference in transcription levels of brain aromatase (*cyp19a2*), *fushi* tarazu-factor 1 (*ftz-f1*), and 11 β-hydroxysteroid dehydrogenase type 2 (*11β-hsd2*) between the SC and NC groups. Exposing fish to Phe-loaded MPs (10 μg/L Phe + 500 μg/L MPs) significantly decreased the transcription levels of *ftz-f1* and *GnRH* (Fig. 7a and b). At the highest concentrations of either virgin MPs or Phe, the transcription levels of *11β-hsd2* were upregulated (Fig. 7c). Both Phe concentrations (either alone or in combination with MPs) downregulated the transcriptional levels of *foxl2* (Fig. 7d). The highest MP concentration (either virgin or Phe-loaded) and the lowest Phe concentration (alone or loaded onto MPs) decreased the transcription levels of *tph2* (Figs. 7e and 7f). Throughout the study, transcript levels of *cyp19a2* were not altered (two-way ANOVA, $P > 0.05$).

4. Discussion

There is a paucity of information about the impacts of MPs on living organisms. Plastic polymers are believed to be inert and, therefore, impose minimal threats to ecosystem health. Accordingly, it is expected that in the event of mixed exposure with other contaminants, it is unlikely that MPs would modulate their toxicokinetics. This study tested these hypotheses by using a suite of biomarker responses in *C. gariepinus*.

Cholesterol is a major component of cell membranes and is a precursor for steroid hormone synthesis (Tewari et al., 1987). High-density lipoproteins play a major role in the transportation and metabolism of cholesterol by removing it from the periphery and delivering to the liver (Norata et al., 2006). Exposure to 50 μg/L virgin MPs significantly reduced the circulating levels of cholesterol and HDL. Liver is the major organ which regulates the metabolism of cholesterol and triglycerides (Barnhart, 1969) and therefore, any damage to the liver could impair circulatory lipid levels in the body (Begriche et al., 2011). Nevertheless, the lack of significant changes in the liver DTC of the fish treated with 50 μg/L MPs compared to control group rejects the idea that liver lesions were responsible for the observed decline in the plasma cholesterol levels. Alternatively, hypocholesterolemia might signal enhanced utilization of cholesterol in corticosteroidogenesis. Although we did not measure plasma cortisol levels in this study, increased cortisol secretion has been a well-documented stress response in different fish species (Kime, 2012; Oliveira et al., 2007). Meanwhile, a decrease in globulin levels is an immunosuppressive response in fish (Atef et al., 1991; Nayak et al., 2004) and therefore, the observed reductions in the plasma globulin level after the exposure to 50 μg/L MPs may be indicative of further immune system suppression to *C. gariepinus*. Lipids and proteins are the major organic compounds in teleosts (Tocher, 2003). Strong correlations between the lipids (i.e., cholesterol, HDL) and proteins (i.e., TP, albumin, globulin) in this study are

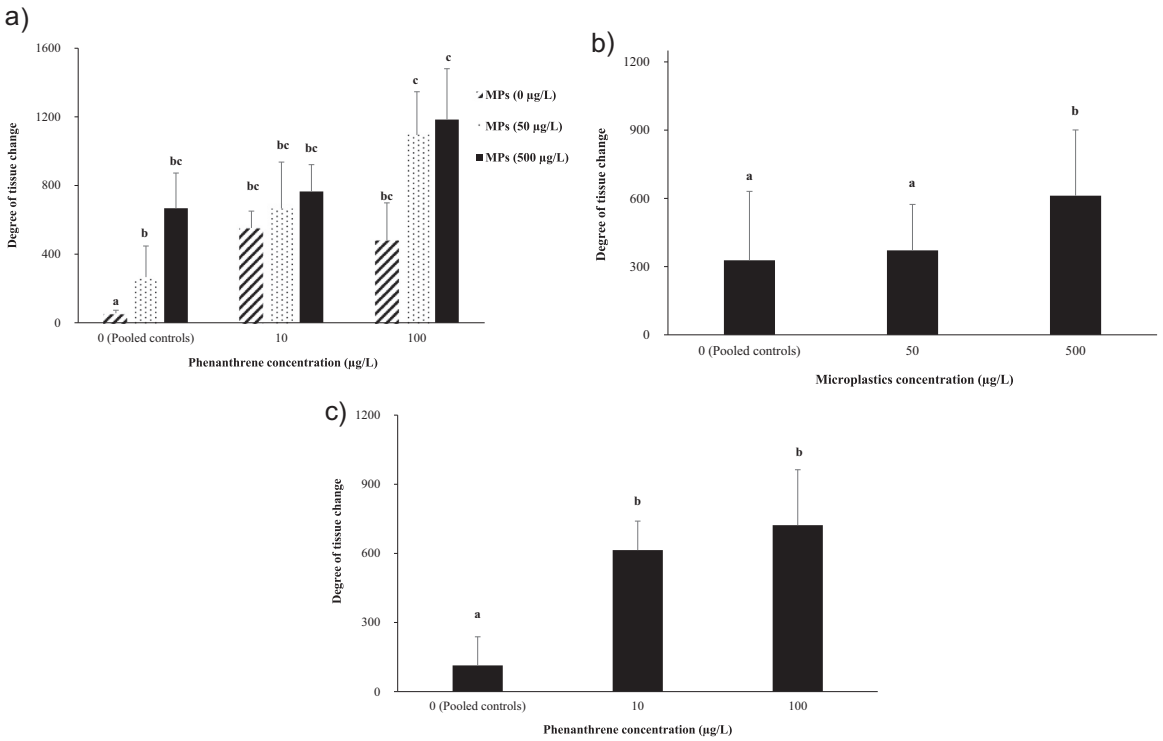

Fig. 2. Degree of tissue change (DTC) in the a) gill of *Clarias gariepinus* following MPs + Phe treatments, and b) and c) in the liver of *C. gariepinus* following MPs + Phe exposure, respectively. Bars labelled with different letter are significantly different (PERMANOVA pairwise comparisons) with Holm–Bonferroni adjusted P-value. In Fig. 2a: n=4 for all the treatments except for the pooled controls (0 µg/L Phe +0 µg/L MPs) where n=8; In Fig. 2b, n=16 for 0 µg/L and n=12 for 50 and 500 µg/L MPs; in Fig. 2c: n=16 for 0 µg/L Phe and n=12 for 10 and 100 µg/L Phe.

consistent with earlier reports on the links between plasma protein and the lipid content in fish following exposure to contaminants (e.g., Gluth and Hanke, 1985). Results of this study showed that the low MP level could potentially be more harmful to the fish health than higher concentrations. The presence of MPs, even at low concentrations, have been reported in even the most remote areas on earth (Free et al., 2014; Obbard et al., 2014). Therefore, more studies are needed to evaluate dose-dependent biological impacts of MPs.

It was surprising to observe biomarker changes in *C. gariepinus* following exposure to virgin LDPE fragments. In accordance with our results, acute exposure to a high concentration (2500 µg/L) of irregularly shaped HDPE particles (0–80 µm in size) induced inflammatory responses such as increasing granulocytoma formation and decreasing lysosomal membrane stability (LMS) in blue mussel (*Mytilus edulis* L.; von Moos et al., 2012). Meanwhile, exposing adult Pacific oyster (*Crassostrea gigas*) to virgin polystyrene microspheres (2 and 6 µm in diameter) decreased D-larval yield and larval development of offspring and shifted energy allocation from reproduction to structural growth (Sussarellu et al., 2016). In the current study, over 20% of particles were sized below 4.3 µm (Appendix D). Initially, we had hypothesized that the observed changes might have been related to the translocation of MPs into the target organs (i.e., liver). However, viewing the liver and gills slides under a polarizing microscope failed to show any traces of MPs within those organs. Undetectable concentrations of major classes of contaminants in the virgin MPs (see section "Concentrations of chemicals on MPs") rejected the possibility that the observed changes were governed by the release of these compounds. The release of ethylene monomers from the LDPE particles under the influence of digestive enzymes and acids, as well as microorganisms might explain the observed changes. The bacterium *Enterobacter asburiae* has shown the ability to biodegrade polyethylene polymers (Yang et al., 2014), which has been reported to be present within the gastrointestinal tracts of freshwater fish species by earlier studies (Mandal and Ghosh, 2013). In turn, the released ethylene could further result in the formation of ethylene oxide and ethylene glycol, which have been shown to render toxic effects in humans and animals (Cox and Phillips, 2004; Grosse et al., 2007). Alternatively, internal and external abrasions caused by the sharp edges of MPs might have initiated a pathway resulting in changes to biomarker responses. In light of these findings, further studies are needed to elucidate the possible mechanisms of virgin PE fragments toxicity in organisms.

The lack of impact of 10 µg/L Phe on circulating levels of cholesterol and HDL compared to the control group is consistent with our recent study on the effects of Phe on biomarker responses in juvenile *C. gariepinus* (Karami et al., 2016). Fish exposed to 100 µg/L Phe had significantly lower blood cholesterol concentrations and higher *11β-hsd2* transcription levels compared to the control group, respectively. The upregulation of *11β-hsd2* might have caused the low circulating levels of cortisol. Low cortisol and cholesterol levels could impair normal behaviour of the fish such as social settings and their responses towards prey and predators and thus compromise the survival rate of a population (Hontela et al., 1992).

Our findings suggested toxicologically relevant interactions between MPs and Phe. The modulating role of MPs on the effects of Phe on plasma lipids in *C. gariepinus* revealed an interesting pattern. The lack of changes in cholesterol and HDL levels following the exposure to MPs (50 or 500 µg/L) loaded with 10 µg/L Phe may suggest that MPs do not modulate the impact of low Phe levels on lipid metabolism, mobilization, and clearance. In contrast, exposure to 500 µg/L MPs loaded with 10 µg/L Phe increased the plasma globulin and TP levels and the content of glycogen-positive material in the liver. Higher globulin levels are believed to be associated with a stronger innate immune responses in fish (Wiegertjes et al., 1996). Interestingly, the increased glycogen

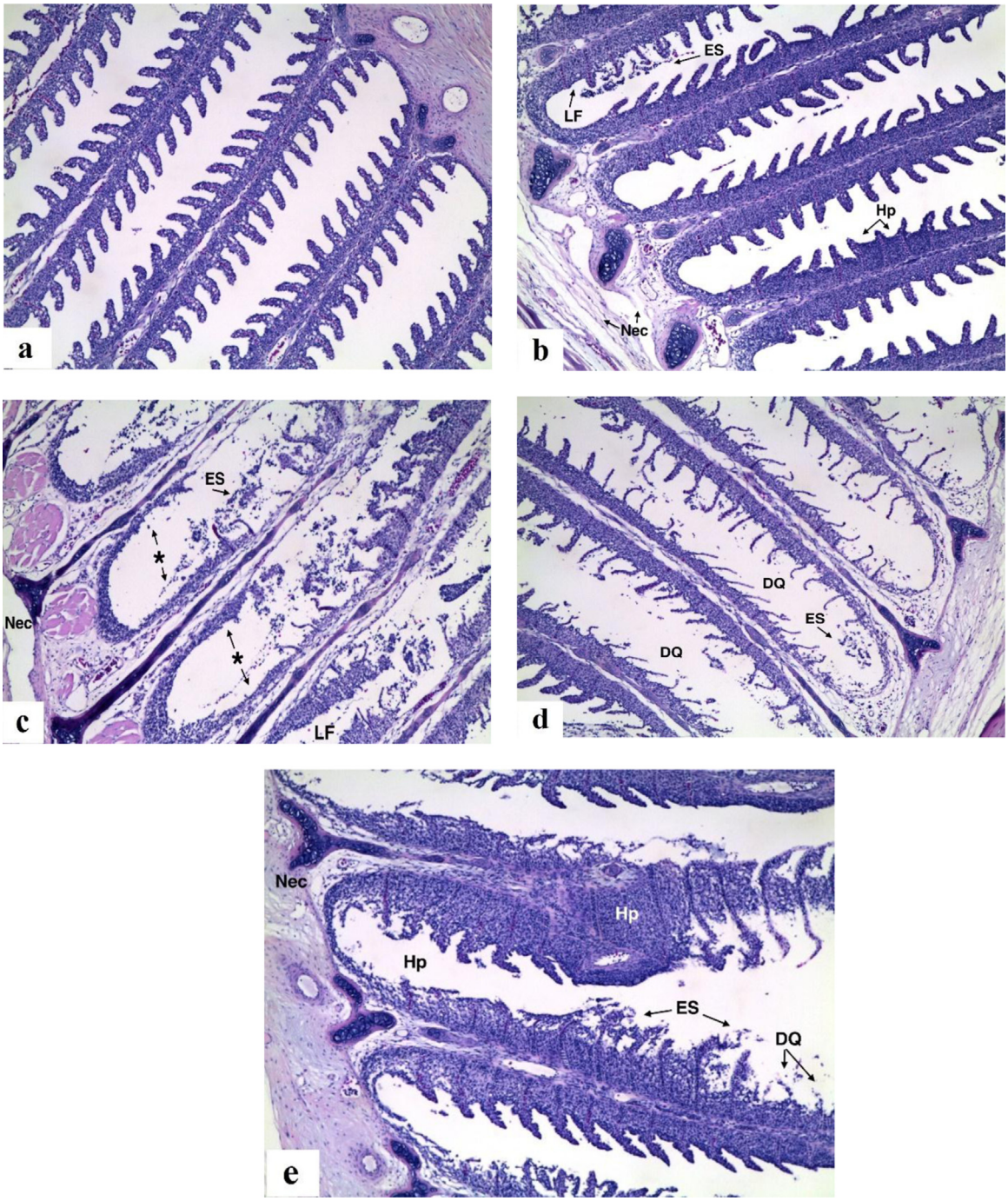

Fig. 3. *Clarias gariepinus* from the control treatment showing normal gill structure with numerous secondary lamellae branching from the primary lamellae (a). However, the secondary lamellae of fish exposed to 50 μg/L MPs (b) showed epithelial sloughing (ES), necrosis (Nec) of the connective tissue and hyperplasia (Hp) that sometimes led to lamellae fusion (LF). In addition to these changes in the 500 μg/L MPs treatment (c), there was also desquamation (DQ) that led to the exposure of the capillaries or even the complete loss of the secondary lamellae (asterisk). For fish in the 100 μg/L Phe (d) there was epithelial sloughing and desquamation, but this was more severe for fish exposed to 500 μg/L MPs loaded with 100 μg/L Phe (e) as well as a greater degree of hyperplasia and secondary lamellae distortion.

stores may indicate a mobilization of glycogen to support the energy required for such an immune response. These results highlight the interference of MPs with Phe toxicity in *C. gariepinus*. Hyperproteinemia is believed to occur due to an osmotic imbalance between extra- and intracellular compartments (Mc Donald and Milligan, 1992). Also, a disruption of normal gill function following exposure to pollutants has been reported in earlier studies (e.g., Lerner et al., 2007). It is possible that the simultaneous exposure to MPs and Phe created lesions in the gill tissue that could have compromised their osmoregulatory abilities, resulting in water loss from the serum and, consequently, increasing the globulin and TP concetrations. Simultaneous exposure to 10 μg/L Phe and 500 μg/L MPs downregulated the transcription levels of *GnRH* and *ftz-f* compared to the control group. Also, the highest concentration of virgin MPs significantly reduced the transcript levels of tph2 compared to the control treatment. Tryptophan hydroxylase (*tph*) is a key player in the hydroxylation of tryptophan into 5-hydroxytryptophan, which is subsequently decarboxylated to serotonin (Walther and Bader, 2003). One of the pivotal functions of serotonin is regulating the transcript level

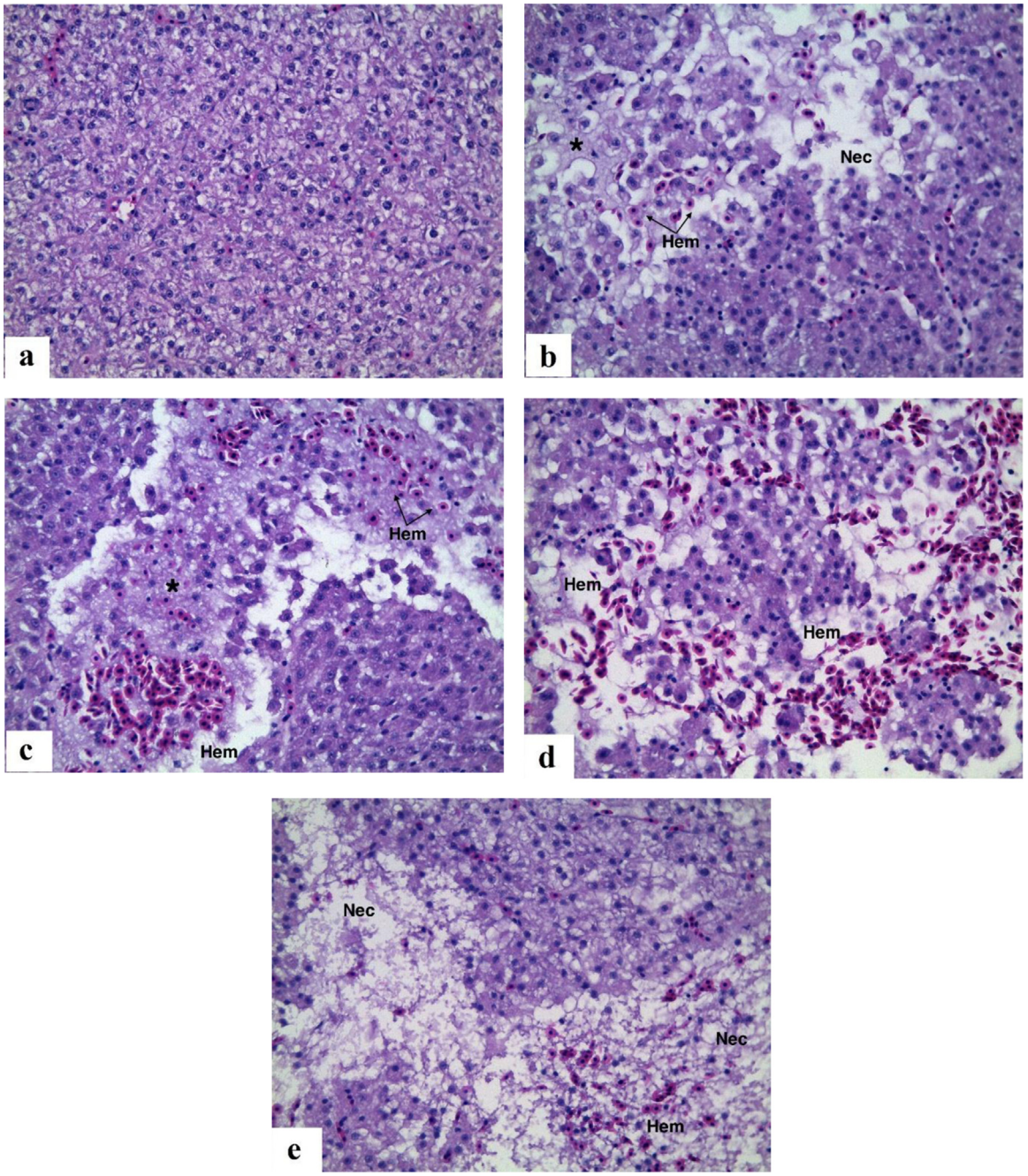

Fig. 4. *Clarias gariepinus* from the control treatment showed normal sinusoid structure and intact hepatocytes (a). In both MP treatments of 50 μg/L (b) and 500 μg/L (c), there were occasional instances of oedema (asterisk), necrosis (Nec) and hemorrhaging (Hem). These changes were generally more intense and frequent for *C. gariepinus* exposed to Phe or loaded onto MPs as seen at 100 μg/L Phe (d) or 100 μg/L Phe loaded onto 500 μg/L MPs (e).

GnRH (Li and Pelletier, 1995). Forkhead box L2 and *ftz-f1* are the transcriptional factors regulating the transcription of brain aromatase gene (*cyp19a2*) in organisms including *C. gariepinus* (Sridevi et al., 2013). Reduced *GnRH* and *ftz*-f1 mRNA levels could further cause a hormonal imbalance and, consequently, affect the reproductive success in fish. In a study by Sun et al. (2015), exposure to 0.06 and 60 μg/L Phe downregulated *GnRH* transcript levels and also inhibited ovarian development and fecundity in female marine medaka (*Oryzias melastigma*). However, no significant interactive effects between MPs and Phe in regulating *foxl2* and *tph2* transcription levels highlighted the selective impact of mixed exposures on the transcription of genes *in C. gariepinus.*

The adsorption of hydrophobic contaminants by MPs could alter the environmental risks and bioavailability of both groups (Chua et al., 2014). Chemical analysis of the Phe-sorbed MPs revealed the ability of MPs to take up Phe from the solution, particularly when the Phe concentration was high (see section "Concentrations of chemicals on MPs"). Acidic condition or the presence surface active digestive substances in the digestive tract of aquatic organisms may facilitate desorption of pollutants from the ingested MPs (Bakir et al., 2014). Therefore, changes in biomarker responses following the exposure to Phe-loaded MPs might be due to the facilitated transportation of Phe into the fish body from the LDPE fragments. Alternatively, the observed changes in biomarker responses might have been due to the microscale abrasions of the gill, skin, and/or intestinal tract caused by the physical impacts of MPs, which facilitated the absorption of higher amounts of Phe. Results of this study should raise awareness of the threats posed

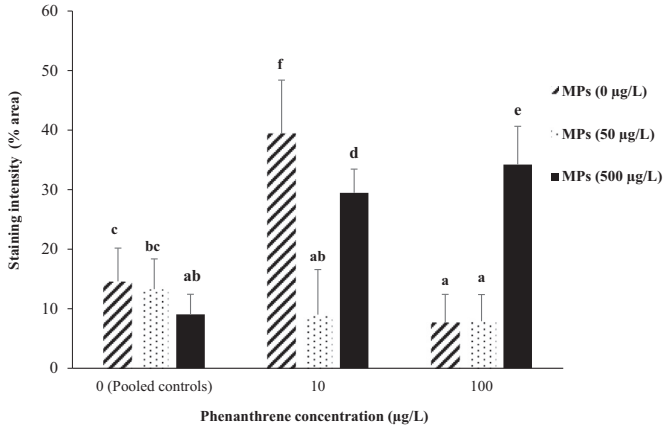

Fig. 5. Periodic acid-Schiff (PAS) stain intensity (% area) in the liver of *Clarias gariepinus*. The results represent means + SD; n=4 for all the treatments except for the pooled controls (0 µg/L Phe +0 µg/L MPs) where n=8. Bars surmounted with different letters are statistically different ($P < 0.05$, Duncan's multiple range test).

by virgin MPs and when loaded with Phe, as they are likely to occur/co-occur in aquatic environments (Cole et al., 2011; Free et al., 2014; Guo et al., 2011; Liu et al., 2009). Further studies using other fish species and larger sample sizes would significantly improve our understanding of the adverse impacts of MPs on the environmental health.

This study also provided a detailed stepwise methodology to investigate exposure to virgin or contaminant-loaded MPs and other contaminants in fish. Factors including insufficient pre-incubation of MPs with the contaminants, lack of chemical analysis of MPs, and a less environmentally relevant choice of MP sources (i.e., microsphere) may increase the risk of committing type I and II errors.

5. Conclusions

Our understanding about the toxicity of MPs in living organisms is evolving. This study showed an acute exposure to virgin LDPE fragments could cause major alterations in some of the

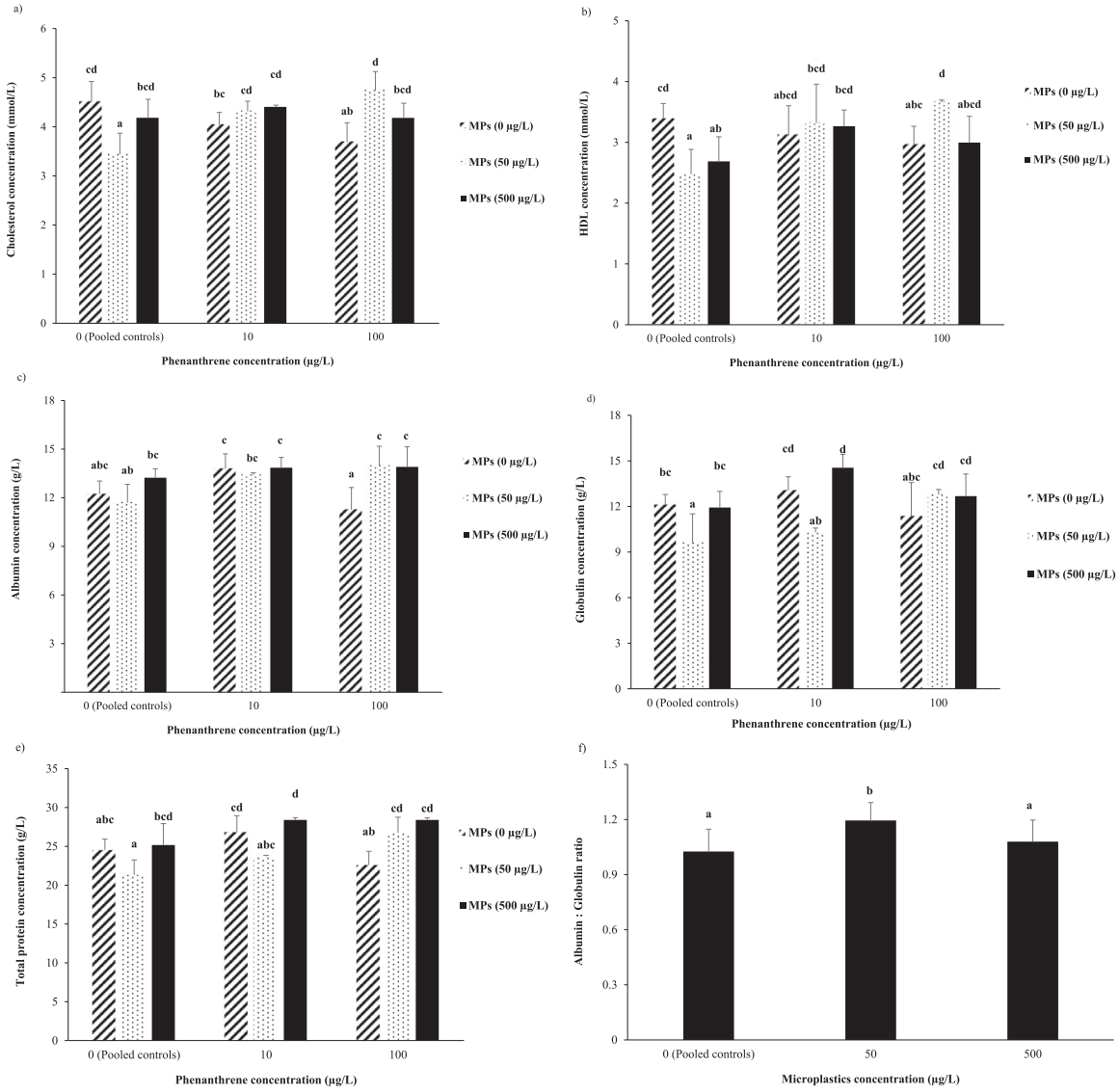

Fig. 6. Plasma a) cholesterol b) HDL c) albumin d) globulin and e) total protein concentrations, and f) albumin: globulin ratio in *Clarias gariepinus*. Bar present means + SD; in panels a-e n=5 for all the treatments except for the pooled controls (0 µg/L Phe +0 µg/L MPs) where n=10; in panel f n=15 for all treatments except the control group where n=20. Bars surmounted with different letters are statistically different ($P < 0.05$, Duncan's multiple range test).

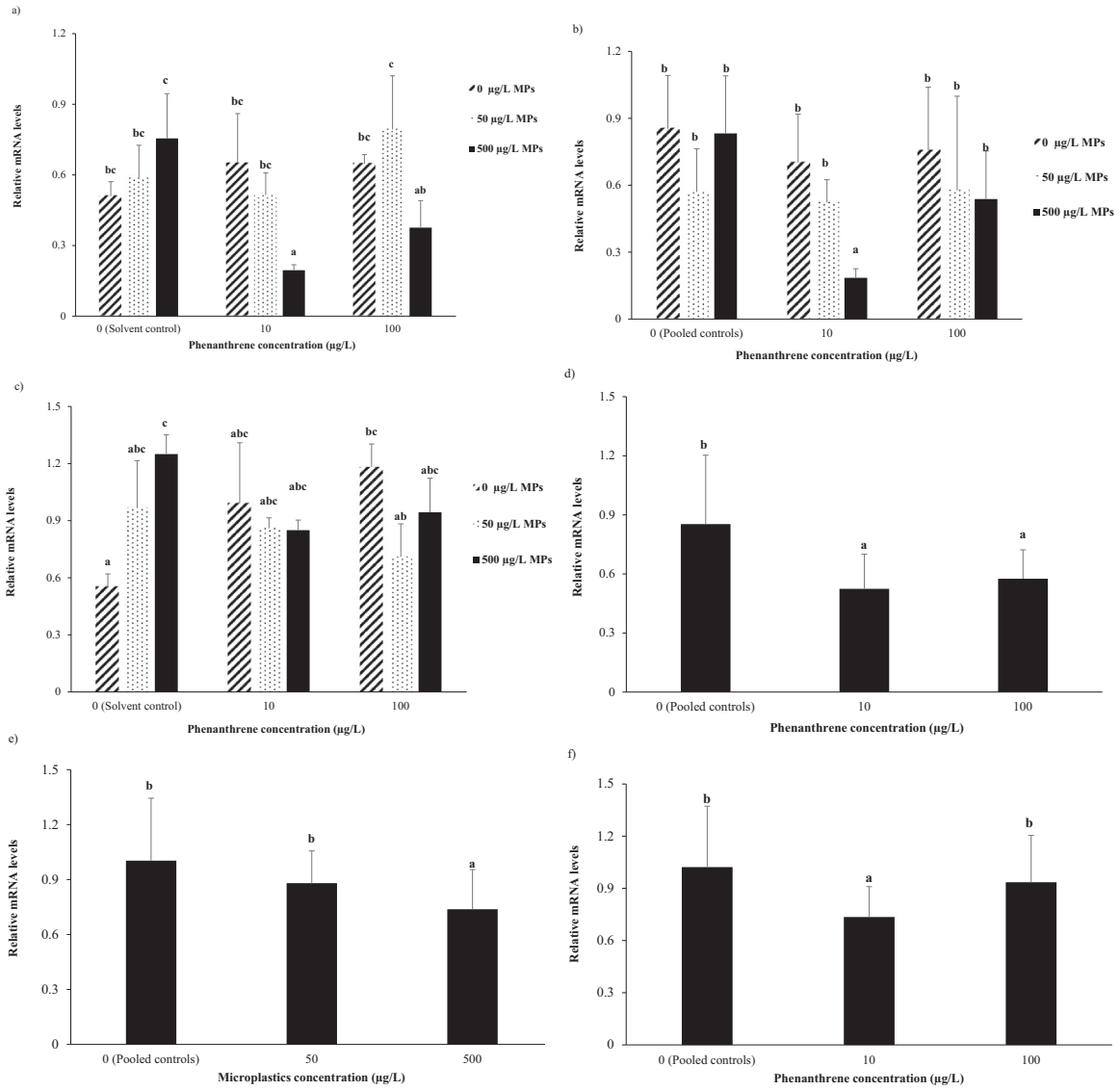

Fig. 7. Relative mRNA levels (mean+SD) of the brain a) *ftz-f1*, b) *GnRH*, c) *11β-hsd2*, d) *foxl2*, and e) and f) *tph2* in the brain of *Clarias gariepinus* across Phe and/or MP treatments; n=5 for all the treatments except for the pooled control treatment where n=10. Relative mRNA level of Negative control=1. Bars labelled with different letters are statistically different from each other ($P<0.05$; Duncan's multiple range tests).

biomarker responses in *C. gariepinus*. These changes do not seem to be due to the presence other contaminants such as PCBs, phthalates, heavy metals or PAHs on MPs. Furthermore, we proved that MPs were able to interact with the effects of Phe on biomarker responses in *C. gariepinus*, possibly by changing its bioavailability. Findings of this study should raise awareness about the ability of virgin or Phe-loaded MPs to influence the physiology of *C. gariepinus*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2016.07.024.

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