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Research Article

Individual and Combined Toxicity of Polycyclic Aromatic Hydrocarbons Phenanthrene and Fluoranthene in Freshwater Amphipod *Gammarus pulex* (L., 1758) (Amphipoda: Gammaridae)

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Abstract: The combined toxicity of polycyclic aromatic hydrocarbons on freshwater amphipod *Gammarus pulex* was investigated. The 14-day LC_{50} values of fluoranthene, phenanthrene and their combination for *G. pulex* were determined as 5.88±0.23, 8.31±0.57 and 7.00±0.33 mg/L, respectively. To evaluate the single and combined toxicity, sublethal concentrations of fluoranthene, phenanthrene and their combination were exposed to *G. pulex* for 14 days. Acetylcholinesterase, glutathione-s-transferase and cytochrome P450 1A1 enzymes were measured as biomarkers. Acetylcholinesterase activities showed significant inhibition at the end of 7 and 14 days. No statistically significant change was observed in cytochrome P450 1A1 levels at the end of the 7th day, but a significant increase was detected at the end of the 14th day. A significant inhibition was observed in glutathione-s-transferase activities at the end of the 7th and 14th days. Statistically significant changes were observed in all parameters when the application times are compared. It was determined that *G. pulex* exposed to different concentrations of phenanthrene and fluoranthene individually and in combination caused significant changes in activites of the all measured enzymes. It was concluded that the cytochrome P450 1A1, glutathione-s-transferase and acetylcholinesterase enzymes in *G. pulex* are useful biochemical markers for the determination of polycyclic aromatic hydrocarbon toxicity.

Key words: Phenantrene, fluoranthene, PAHs, *Gammapus pulex*

Introduction

Polycyclic aromatic hydrocarbons (PAHs), a class of toxic xenobiotics resulting from the incomplete burning of organic compounds with two or more fused benzene rings, have toxic and carcinogenic effects (Zhang et al. 2020). Fluoranthene (FLU) is one of the EPA's 16 PAHs identified as a priority pollutant and found abundant in many countries (Poor et al. 2004). FLU has been shown to be acutely toxic to some aquatic organisms (USEPA 1978, 1993). Since phenanthrene (PHE) is widely distributed in the environment, it easily accumulates in aquatic organisms and causes the activation of detoxification and biotransformation mechanisms (Bebianno & Barreira 2009, Boutet et al. 2004).

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AChE has been used as a useful neurotoxicity biomarker of exposure of living organisms to pollutants in various environments (Amiard & Triquet 2009). The toxicity of FLU and PHE is mainly due to the inhibition of AChE activity, which interferes with the function of the nervous system by leading to the accumulation of acetylcholine (WHO 1986). *G. pulex* can eliminate PAHs in environments through a detoxification process with CYPs and then with GSTs (Jørgensen et al. 2008). CYP enzymes are some of the key enzymes in the CYP450 reaction cycle the detoxification systems of virtually all living organisms and are subdivided into different gene-families, some of them which are induced by coplanar (flat) contaminants such as PAHs, PCB´s and dioxins (Rewitz et al. 2003, Rust et al. 2004). A way of measuring exposure of a contaminant is determining the activity of CYP1A in anorganism (Petersen et al. 2012). GST is a family of enzymes responsible for the metabolism of many xenobiotic, cytotoxic agents and carcinogens (Rodrigues et al. 2015, CAMPOS et al. 2016). GSTs prevent toxic foreign substances taken from the outside to combine with other macromolecules in the body and allow these compounds to be removed without damaging cell components (Bulavin et al. 1996, Aksu et al. 2017).

Most of the studies on xenobiotics it was investigated that what kind of effects would occur when pollutants were used alone but pollutants are not found alone in nature, they are found together with one or more pollutants (Van Wezel & Opperhuizen 1995). In ecosystems, organisms are exposed to a wide variety of toxic substances instead of a single chemical (Yang et al. 2017). The antagonistic or synergistic effects of such mixtures of pollutants depend on the toxic action mechanisms of each, and it is difficult to determine the combinatorial toxic effects of these mixtures (Nagai 2017,Li et al. 2017, Yang et al. 2017).

Gammarids are organisms commonly used in aquatic toxicology to evaluate the effects of PAHs in freshwater ecosystems (Rinderhagen et al. 2000). They are active throughout the entire food chain. Therefore, these species are often preferred as test organisms because they can be sampled by collecting throughout the year and they can be easily identified, controlled, and maintained in the laboratory conditions (Uğurlu et al. 2015).

The aim of this study was to determine the toxicity of FLU, PHE and their combination in *G. pulex* using CYP1A1, GST and AChE enzymes as biomarkers.

Materials and Methods

Organisms

G. pulex (L., 1758) used as a model organism in present study were obtained from stock cultures which are maintained at the University of Munzur. The cultured *G. pulex* were brought alive to the Aquatic Toxicology Research Laboratory where the experimental application will be made and kept for 15 days for adaptation to these conditions. *G. pulex*, which was brought alive to the laboratory for experimental study, was selected from healthy male individuals of similar size.

Exposure Media

The temperature and lighting of the laboratory has been controlled. In the lighting, a photoperiod of 12:12 light : dark was applied with a timed electrical circuit. The temperature was set to 18±0.5 °C in all experimental stages with a thermostatic air conditioner. An external filter was used for water circulation in stock aquariums. During the adaptation period, the feeding and mobility of the living things were observed.

Experimental Design

Methanol was used as a carrier solvent to prepare the PHE and FLU stock solutions. The maximum amount of methanol in the final test or exposure medium foe each PAHs was ≤ 0.1 mL L^{-1} to avoid possible confusing effects of the carrier. The acute toxicities of the PHE and FLU used in the study, individually and in combination, were determined by the 96-hour static LC_{50} test. In acute toxicity experimental studies, organisms were not fed within 96 hours. During the experiment, each concentration group was observed at 24, 48, 72 and 96 hours. Dead Gammarus individuals were taken from the experimental media, noted and recorded. 96-hour LC_{50} toxicity values were calculated using the probit analysis from the data obtained.

The application groups containing the sublethal concentration of FLU, PHE and FLU+PHE, which were determined by the ratio of 1/40, 1/20 and 1/10 of the LC_{50} value. The test organisms were exposed to:

- 1/40 of the LC_{50} value of PHE in Group A;
- 1/20 of the LC_{50} value of PHE in Group B;
- 1/10 of the LC_{50} value of PHE in Group C;
- 1/40 of the LC_{50} value of FLU in Group D;
- 1/20 of the LC_{50} value of FLU in Group E;
- 1/10 of the LC_{50} value of FLU in Group F;
- 1/40 of the LC_{50} value of FLU+PHE in Group G;
- 1/20 of the LC_{50} value of FLU+PHE in Group H;

Table 1. LC_{50} values of PHE, FLU and FLU + PHE for *Gammarus pulex*

PAHs	LC_{50} (mg/L)
FLU	5.88±0.23
PHE	8.31±0.57
FLU + PHE	7.00±0.33

- 1/10 of the LC_{50} value of FLU + PHE in Group I.

The seven *Gammarus* individuals were exposed to these application groups for 7 and 14 days in 1-L glass containers. After exposure, the test organisms were rapidly stored in an ultra-freezer at -86 ˚C until biochemical analysis was performed.

Biochemical analyses

In this study, 0.5 g of Gammarus individuals were weighed and 1/5 w/v ratio of PBS buffer was added and homogenised with ice. These homogenised samples were centrifuged at 17.000 rpm for 15 min in a 4°C refrigerated centrifuge and the supernatants kept at -86 °C until biochemical assay.

GST, CYP1A1 and AChE activities were analysed to determine the individual and combined toxicity of PAHs. Enzyme-Linked Immunosorbent Assay (ELISA) kits were used for the analysis of all biochemical parameters and measurements were made with Microplate Reader, Thermo Scan FC. The GST kit was purchased from Cayman Chemical (Ann Arbor, MI, USA), CYP1A1 and AChE kits were purchased from Cusabio Biotech Co., Ltd. (Wuhan, China). Catalog numbers are GST: 703302, CYP1A1: CSB-EL006395FI, AChE: CSB-E17001Fh respectively.

Statistical Analysis

Statistical analyses were carried out using PASW Statistics 18 software. The LC_{50} value of each drug in *G. pulex* was calculated using probit analysis. Duncan's multiple range test at $P<0.05$ level was used to determine the statistical differences amoung groups in the same application periods. The statistical differences between the application periods (7 and 14 days) were determined by two-tailed independent-samples t test.

Results

LC_{50} values

LC_{50} values of PHE, FLU and FLU + PHE for *G. pulex* were shown in Table 1.

Biochemical parametres

For the CYP1A1, GST and AChE activities in *G. pulex* exposed to application groups, see Fig. 1, 2 and 3.

AChE activities

It was observed that a statistically significant inhibition in AChE activities in groups A, D and I at the end of the 7th day compared to the control group

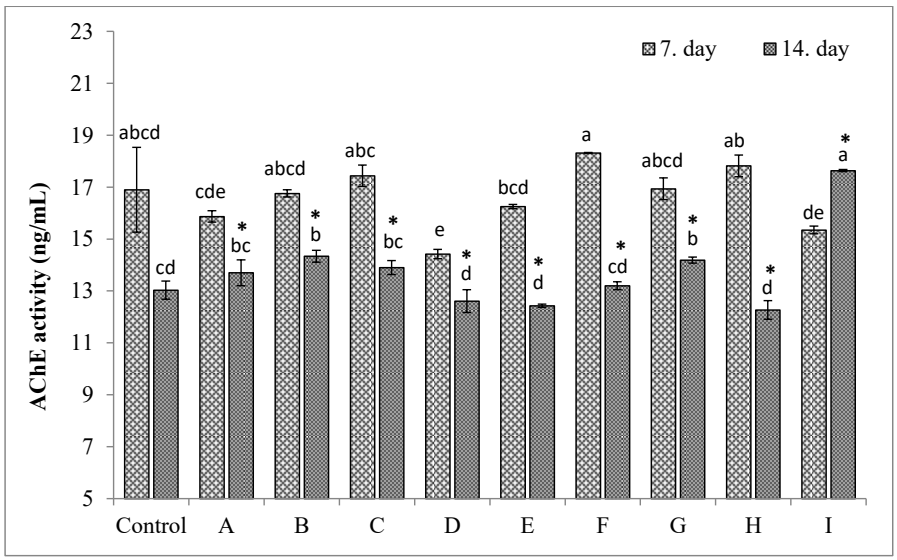

Fig. 1. Changes in AChE activities in *G. pulex* in control and application groups. The different letters (a, b, c) on the bars indicate the significant differences among application groups (control, A, B, C, D, E, F, G, H, I) at the same exposure times (7 or 14 d) according to the Duncan's multiple range test. The asterisk (*) on the bars indicates the statistical differences between the exposure times (7 and 14 d) in the same application group according two-tailed independent-samples t test.

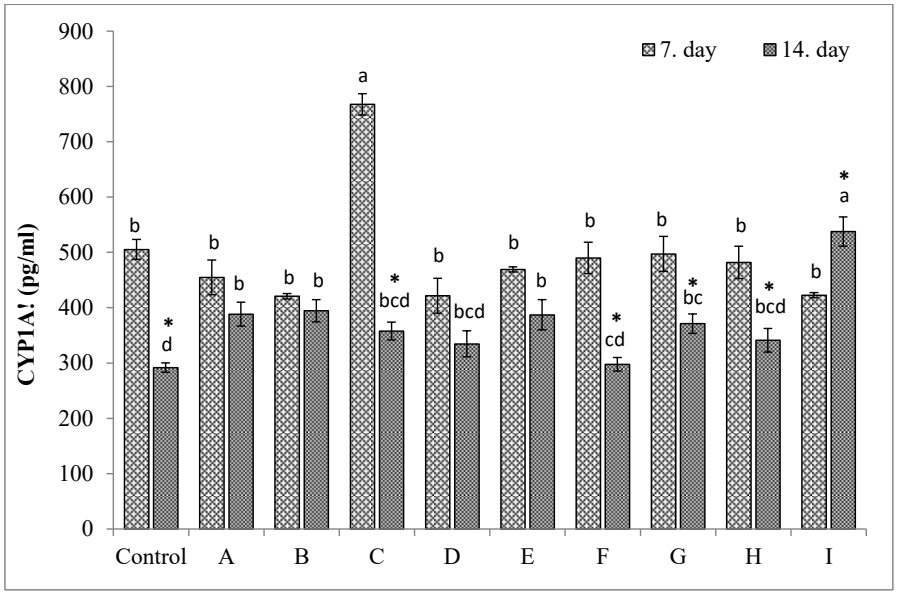

Fig. 2. Changes in CYP1A1 in *G. pulex* in control and application groups. The different letters (a, b, c, d) on the bars indicate the significant difference among application groups (control, A, B, C, D, E, F, G, H, I) at the same exposure times (7 or 14 d) according to the Duncan's multiple range test. The asterisk (*) on the bars indicates the statistical differences between the exposure times (7 and 14 d) in the same application group according two-tailed independent-samples t test.

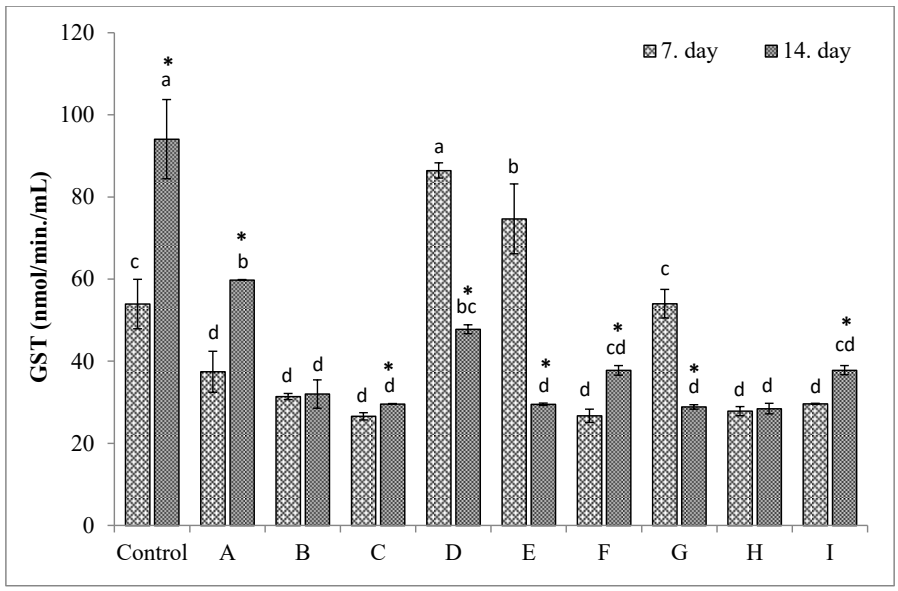

Fig. 3. Changes in GST activities in *G. pulex* in control and application groups. The different letters (a, b, c) on the bars indicate the significant difference among application groups (control, A, B, C, D, E, F, G, H, I) at the same exposure times (7 or 14 d) according to the Duncan's multiple range test. The asterisk (*) on the bars indicates the statistical differences between the exposure times (7 and 14 d) in the same application group according two-tailed independent-samples t test.

($p<0.05$). Smilarly, an inhibition was observed in AChE activities in groups D, E and H compared to the control group at the end of the 14th day ($p<0.05$). It was determined that the highest inhibition was in the H group where FLU + PHE were exposed ($p<0.05$). When the exposure times were compared, it was observed that there was a statistically significant decrease in the enzyme activities on the 14th day in all application groups except I group ($p<0.05$) (Fig. 1).

CYP1A1 activities

No statistically significant change was observed in CYP1A1 enzyme activities in all application groups at the end of the 7th day compared to the control

group ($p>0.05$). A statistically significant increase was found in these enzyme activities at all application groups compared to the control group at the end of the 14th day ($p<0.05$). When the exposure times were compared, CYP1A1 activities were statistically significantly decreased in all application groups except the groups A, B and E ($p<0.05$) (Fig. 2).

GST Activities

A statistically significant inhibition in GST activities was observed in the groups A, B, C, F, H and I at the end of the 7th day compared to the control group ($p<0.05$). Smilarly, an inhibition in these enzyme activities was observed in all treatment groups at the end of the 14th day, compared to the control group ($p<0.05$). When the exposure times were compared, an increase in GST activities was observed in the A, B, C, F, H, I groups, while a statistically significant decrease was found in the D, E and G groups ($p<0.05$) (Fig. 3).

Discussion

PAHs are chemicals compounds that produced by incomplete combustion of fossil fuels. They are found in petroleum and petroleum derivatives. Many of the PAHs cause pollution and significantly affect the biological balance due to their persistent structure. The PAHs destruction has done in the ecological balance because of their transport and accumulation in the environment has become an important problem all over the world over the years (Bartonitz et al. 2020).

In various studies, LC_{50} values of different polyaromatic hydrocarbons have been determined for different model organisms. Sanz–Lazaro et al. (2008) found that the 48h-LC_{50} of PHE was 173.85 and 147.64 µg/L for *Gammarus aequicauda* and *Gammarus locusta* respectivelly. The 48h-LC_{50} of fluoranthene was 49.99 and 42.71 µg/L for *G. aequicauda* and *G. locusta*, respectivelly. Acute toxicity data (LC_{50} or EC_{50} values) of PHE, after 48 h exposure, was: *Nitzschia palea* – 870 µg/L; *Pseudokirchneriella subcapitata* – 233 µg/L, *Scenedesmus vacuolatus* – 590 µg/L, *Daphnia magna* – 700 µg/L; *D. pulex* – 100 µg/L, *Diporeia* spp. – 74 µg/L, *Gammarus minus* – 460 µg/L, *Anabaena flos-aqua* – 1300 µg/L and *Chironomus riparius* – 41 µg/L. The acute toxicity data of fluoranthene (LC_{50} or EC_{50}) to freshwater species was 35 µg/L for *Scenedesmus vacuolatus*, 111 µg/L – *Rana catesbeiana*, 2.0 µg/L – *R. pipiens*, 193 µg/L – *Xenopus laevis,* 1.2 µg/L – *Lumbriculus variegatus*, 2.2 µg/L – *Hydra americana*, 45 µg/L – *Ceriodaphnia dubia*, 1.6 µg/L – *Daphnia magna*, 108 µg/L – *Gammarus pseudolimnaeus* and 183 µg/L – *Hyalella azteca* (Verbruggen 2012). In present study, the LC_{50} values of FLU, PHE and FLU + PHE for *G. pulex* were 5.88±0.23, 8.31±0.57 and 7.00±0.33 mg/L, respectivelly.

PAHs are metabolised by various xenobiotic metabolising enzymes such as CYP450, epoxide hydrolase, glutath ione s-transferase, UDP-glucuronosyltransferase and sulfotransferase. These enzymes are mainly involved in the conversion of PAHs to polar water-soluble metabolites, and the resulting metabolites are easily excreted from the body (Shimada 2006).

Kim et al. (2018) evaluated single and combined toxicities of endosulfan and PHE using zebrafish (*Danio rerio*) adults. They determined that combination of endosulfan and PHE exhibited a synergistic effect. They also found that when zebrafish was exposed to endosulfan alone or in combination with PHE, AChE activity decreased, GST activity was higher at combined exposure, and CYP1A gene expression increased in all groups. Khpalwak et al. (2018) investigated single and combined effects of FLU, PHE, mannitol and sulfuric acid using fumigation method for 40 days on *Calendula officinalis* seedlings. Their results revealed that FLU fumigation induced oxidative stress in plants through the production of ROS. Ertl et al. (2016) tested the molecular effects of the combined exposure of FLU and pyrene for 7 days on *S. glomerata* using RNA-Seq. They found that some transcripts of genes involved in PAH detoxification (e.g. cytochrome P450) were induced in response to the stressors. Wu et al. (2012) determined that the combination of PHE and pyrene at higher concentrations caused changes in antioxidant enzyme activities, detoxification functions and damage levels of earthworms (*Eisenia fetida*) with a synergistic effect. Gravato & Guilhermino (2009) investigated the effects of benzo(a)pyrene on seabass (*Dicentrarchus labrax*) juveniles. They suggest that ethoxyresorufin O-deethylase and GST activities increased in liver of seabass. Zhang et al. (2021) evaluated the individual and combined effects of PHE and polystyrene microplastics on oxidative stress in the clam *Mactra veneriformis*. They found that PHE or polystyrene microplastics could induce oxidative stress to clams. Besides, exposed to 50 µg L^{-1} PHE or 150 µm polystyrene microplastics caused the reduced expression of GST activities, leading to potential oxidative injury in clams. Filipović et al. (2019) investigated the effect of chronic exposure to environmentally relevant concentrations of FLU on defense mechanisms of the polyphagous forest insects *Lymantria dispar* L. and *Euproctis chrysorrhoea* L. They found increased GST activity in the midgut tissue of *E. chrysorrhoea* larvae. In another study conducted by Rodrigues et

al. (2013), increases in GST activity were also found in *Carcinus maneas*, which was exposed to 16–100 µg L^{-1} floranthene for 7 days. Similarly, Baussant et al. (2009) showed an increase in GST activity as a result of exposure of *Mytilus edulis* to crude oil. In a study conducted by Zhang et al. (2019) single and combined effects of microplastics and roxithromycin on *D. magna* were investigated. They determined that exposure to microplastics or roxithromycin alone for 48 h increased GST activities, while combined exposure decreased GST activity. In a study conducted by Yuan et al. (2021) it was determined that GST activity increased in order to provide conjugation with metabolites formed as a result of phase 1 reactions, depending on the increasing concentration of benzo[a]pyrene. In present study, a significant inhibition was observed in GST enzyme activities compared to the control group at the end of the 7th and 14th days. It is thought that this decrease in GST activity may be related to the competition between the metabolites formed as a result of PAH biotransformation and the endogenous substrates (Yao et al. 2017). Turja et al. (2020) evaluated the biochemical responses and accumulation of PAHs in *Gammarus oceanicus*. They observed that GST activity in *G. oceanicus* was unchanged after 7 days of exposure compared to the control. In present study, no statistically significant change was observed in CYP1A1 levels at the end of the 7th day but a statistically significant increase was detected at the end of the 14th day compared to the control group. It can be suggested that enzymes involved in defense may play different roles in the detoxification (Kim et al. 2018). A significant inhibition was observed in GST enzyme activities compared to the control group at the end of the 7th and 14th days suggesting that GST plays a key role in PAH metabolism in gammarids. Overproduction of ROS may inhibit GST activation (Fernandes et al. 2009). Smilarly, Rabechi et al. (2021) investigated the effects of sub-lethal concentrations of PHE on *Chironomus sancticaroli* larvae. They indicated that PHE at 0.0025, 1.25 and 2.44 mg L^{-1} inhibited GST activity.

Exposure to PAHs has been shown to cause neurotoxic effects by some mechanisms, such as inhibition of AChE enzyme activity (Maisano et al. 2017). In the study conducted by Santos et al. (2021) zebrafish (*Danio rerio*) were exposed to microplastics, two sub-lethal concentrations of copper and their mixtures until 14-days post-fertilization. Single or combined exposure to microplastics and Cu caused neurotoxicity in larvae by inhibiting AChE activity. Oliveira et al. (2013) investigated single and combined effects of microplastics and pyrene on juveniles of the common goby *Pomatoschistus microps*. They found that microplastics alone, in combination with pyrene, or alone pyrene significantly reduced AChE activity. Szczybelski et al. (2019) evaluated the use of AChE and GST as potential biomarkers after exposure to pyrene and two pyrene metabolites (1-hydroxypyrene and pyrene-1) in *Astarte borealis*. They indicated that a reduction in AChE levels was considered potentially indicator of the stressful physiological state of *A. borealis*. Rabechi et al. (2021) found that AChE activity singularly was inhibited 24.3% at 2.44 mg L^{-1} in sub-lethal concentrations of PHE exposed *Chironomus sancticaroli* larvae. In present study, a statistically significant inhibition in AChE activity was observed in groups A, D and I at the end of the 7th day when compared to the control group ($p< 0.05$). At the end of the 14th day, inhibition in AChE activity was observed again in the D, E and H groups compared to the control group ($p<0.05$). It was determined that the highest inhibition in AChE activity was in the H group, in which PHE + FLU were exposed ($p<0.05$). The reason for more inhibition at AChE levels than when they are applied individually can be explained by the additive and synergistic interaction, which means that when chemicals are used together, they increase the effect of each other.

Conclusion

In conclusion, it was determined that *G. pulex* exposed to different concentrations of PHE and FLU polycyclic aromatic hydrocarbons individually or in combination, caused statistically significant changes in CYP1A1, GST and AChE enzyme activities. The biochemical response of *G. pulex* to studied PAHs varied depending on the exposure time, the concentration of PHE and FLU, and combined or single exposure. It was concluded that CYP1A1, GST and AChE, which are the biomarkers we used to investigate the toxic effects of PHE and FLU on *G. pulex*, are useful biomarkers. It was also determined that PHE and FLU can pose an environmental threat, and *G. pulex* is a suitable model organism to evaluate the toxic effects of polycyclic aromatic hydrocarbons in aquatic environments. It has been observed that the effect of PHE and FLU on some biochemical parameters is more in the combined exposure compared to the individual exposure. Toxic chemicals interact with other chemicals in the environment to form new compounds, chelates, complexes and their toxicity may change. Further studies are needed on the possible combined effects of polycyclic aromatic hydrocarbons on living organisms to assess the ecotoxicological risk.

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Authors' contributions: OS, NCY and TD designed and carried out the research. OS and NYC wrote the original draft. OS, NCY and TD analysed data. All authors contributed to the revision of the manuscript.

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