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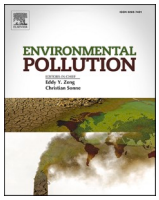

Synergistic, antagonistic, and additive effects of naphthalene, phenanthrene, fluoranthene and benzo(k)fluoranthene on *Artemia franciscana* nauplii and adult☆

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are widespread across the globe and can be highly toxic for the marine environment. This research investigated the short-term (48 h of exposure) effects of PAHs mixtures on the nauplii and adult of crustacean *Artemia franciscana* considering the impact in term of toxicity and changes in gene expression. Results showed that all combinations caused additive or synergic effects with the exception of naphthalene + phenanthrene (NAP + PHE; Combination Index (CI) = 22.3), while naphthalene + benzo(k) fluoranthene (NAP + BkF; CI = 7.8) mixture evidenced an antagonistic effect. Real-time qPCR showed that all mixtures impacted the expression level of the five known genes involved in *Artemia* stress response. The effects of PAHs at environmental concentrations on both adult and nauplii suggested the need for further investigations about the impact of such contaminants on the marine biota considering that crustaceans can accumulate PAHs at concentrations comparable to those assessed in the present study.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of over 100 different ubiquitous organic compounds, highly toxic and predominantly generated by anthropogenic activities (about 99%). Considering the rise of PAHs levels in the marine environment (Medeiros and Caruso Bícego, 2004; Pedrazzani et al., 2019; Ghosal et al., 2016), they were included in the priority list of the EU Water Framework Directive 2000/60/EC (Borja et al., 2004). PAHs are not very soluble in water and are resistant to biodegradation; their concentration in the water column remains stable for a long time, causing potential great problems for the biota and marine invertebrates in particular (Albarano et al., 2022b, 2021a; 2021b; Ben Othman et al., 2023; Gregorin et al., 2021; Honda and Suzuki, 2020; Sun et al., 2021). Specifically, PAHs with low-molecular-weight (*i.e.* naphthalene, fluorene, acenaphthene, and phenanthrene) and PAHs with high-molecular-weight (*i.e.* pyrene, chrysene, benzo[a]pyrene, and dibenz[a,h]anthracene) present half-lives ranging approximately from 3 to 8 days and 73–1780 days, respectively (Shi et al., 2020; Tansel et al., 2011).

To understand the impact of PAHs on aquatic environment, numerous studies have been conducted considering various marine organisms highlighting toxic effects not only on survival, but also on bioaccumulation, embryonic development, and genotoxicity (Albarano et al., 2022b, 2022a; 2021a, 2021b; Balcıoğlu, 2016; Gregorin et al., 2021; Honda and Suzuki, 2020; Noh et al., 2018; Suzuki et al., 2015; Zheng et al., 2020).

Under realistic scenarios such as industrial oil spills, PAHs pollution generally occurs as a mixture of different PAH-compounds. However, the current knowledge about toxicity of PAHs to marine species focuses primarily on some specific PAHs such as pyrene, naphthalene, anthracene, benzo[b]fluoranthene, benzo[a]pyrene, phenanthrene, and chrysene (Sun et al., 2021).

The adverse effects of PAHs mixtures on marine crustaceans still remain largely unknown, let alone the impact of their interactions with

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other emergent pollutants such as microplastics (MPs), heavy metals and drugs. Few studies investigated how PAHs can affect survival in crustaceans, especially in *Artemia franciscana* that is a model organism of interest for investigating gene expression (Albarano et al., 2022b, 2022a; Rocha et al., 2021; Sukumaran and Grant, 2013a, 2013b).

The aim of this research paper was to determine the *in vitro* combined effects both in nauplii and adults of *A. franciscana* of binary, tertiary and quaternary mixtures of naphthalene (NAP), phenanthrene (PHE), fluoranthene (FLT) and benzo(k)fluoranthene (BkF) (already tested separately in (Albarano et al., 2022b)) considering mortality and the expression variation of five stress response genes as endpoints.

2. Materials and methods

2.1. Hatching and toxicological trials

Mortality after 48 h of exposure to PAHs mixture were performed using both *A. franciscana* nauplii and adult according to standard methods (CNR, 2003). The experimental procedures for the hatching cysts and incubation of organisms with different PAHs mixture were in accordance to (Albarano et al., 2022b). Based on the literature information of PAHs concentrations in polluted sediment used in tests with crustaceans and other organisms (*i.e.* Bellas et al., 2008; Arienzo et al., 2017a; Albarano et al., 2022b; Nagyová et al., 2022), the 24-well plates were filled with test solutions that contained nominal concentrations (in μg/L) of naphthalene, phenanthrene, fluoranthene and benzo-k-fluoranthene (all details about exposure concentrations of the mixtures were reported in Supplementary Materials and Table S1 and are based on mixture ratios of the single substances calculated as EC_{50}). The nominal concentrations were determined according to ((Carotenuto et al., 2020); see Table S2). Specifically, PAHs into seawater samples were determined by solid-phase extraction (SPE), filtered and pre-concentrated on a C18 disk (ENVI, −18 DSK SPE Disk, diam. 47 mm). Analytes were eluted with a solution of 1:1 dichloromethane and n-hexane. The extract was then concentrated to 1 mL in Multivap under nitrogen flow (Multivap8, LabTech, Italy). The extract was injected into a gas chromatography-mass spectrometer (GC-MS, MS-TQ8030-Shimadzu, Japan). The limits of detection (LOD) and quantification (LOQ) were calculated, and the average values for the seawater samples were 0.05 μg/L and 0.01 μg/L, respectively.

Six binary and four tertiary mixtures, and a total mixture of four PAHs were designed to investigate the toxic interaction of these organic substances mixture using Combination Index (CI) (Jonker et al., 2005). Individual inhibitory effects of each PAHs (LC_{50} reported in Albarano et al., 2022a) were used for the quantification of synergism or antagonism for n-substances combination at x% inhibition according to (Jonker et al., 2005; McCarty L.S. et al., 1992; Pape-Lindstrom and Lydy, 1997):

$$^{n}(CI)_{50} = \sum_{j=1}^{n} \frac{(D)_j}{(D_{50})_j} \qquad \text{Equation (1)}$$

where $^{n}(CI)_{50}$ represents the combination index for n substances at 50% inhibition, $(D)_j$ is the sum of the dose of n chemicals that exerts 50% inhibition in combination, $(D_{50})_j$, is the dose of each substance taken alone exerting 50% inhibition. From Equation (1), the resulting CI value can be CI < 1, CI = 1, and CI > 1 indicating synergistic, additive, and antagonist effects, respectively.

All details about statistical analyses were reported in Supplementary Materials.

2.2. Gene expression by real time qPCR

Four hundred nauplii and ten adults were exposed to binary, tertiary and quaternary mixtures at different concentrations ratios (see Table S3). Collection of nauplii, adult at 48h and total RNA extraction and cDNA synthesis were performed according to (Albarano et al., 2022b). All details were reported in Supplementary Materials.

The expression levels of 5 genes were followed by real-time qPCR together with *GAPDH* (Chen et al., 2009) used as a control gene to internally normalize the data using REST software (Relative Expression Software Tool, Weihenstephan, Germany; version 1.9.12) (Pfaffl, 2001; Pfaffl et al., 2002). Relative expression ratios greater than ±1.5 were considered as significant. The nonparametric Mann–Whitney test was applied to ΔCq (Cq gene of interest—Cq reference) values between treated and control samples (n = 3) (p-Values <0.05 were considered significant). Statistical analyses were performed using GraphPad Prism Software (version 9.00 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com, accessed on1 May 2022). Fold-change values were represented through a Heatmap generated by GraphPad Prism Software.

3. Results

3.1. Mixture toxicity

To determine the effects of binary, tertiary and quaternary mixtures on *A. franciscana* nauplii and adults' survival, we used the concentrations that induced approximately 50% when these PAHs were tested individually (Albarano et al., 2022b) (Table S1).

3.1.1. Binary mixture toxicity

After nauplii exposure to NAP + PHE mixture, a statistically significant increase of about 30% in toxicity was observed respect to the control ($p < 0.0001$) and the lowest ratios (1:1, 1:2, 1:3, 2:1, 2:2 and 4:1 ($p < 0.01$), and 3:1 ($p < 0.0001$)) when NAP concentration was increased by 2–6 times and PHE concentration was increased by 6–10 times (Fig. S1A). When NAP concentration was increased by 7–8 times and PHE concentration was increased by 2–10 times, a mortality of about 40–50% with respect to control ($p < 0.0001$) was induced (Fig. S1A). By considering the case in which NAP amount was increased by 9–10 times and PHE concentration was increased by 2–10 times, we observed a statistically significant increase in mortality ranging from 50 to 75% respect to the control and lowest ratios ($p < 0.0001$; Fig. S1A). As reported in Fig. S1B, after adult exposure to the same mixture, we observed a statistically significant increase of about 40% in toxicity respect to the control and ratio 1:1 ($p < 0.0001$) only when both concentrations have been doubled.

After exposure to NAP + FLT mixture (Figs. S1C–D), the scenario for nauplii was similar to the one showed for adults. In particular, when the concentration of the FLT was decreased from 1/2 to 1/4 and that of the NAP up to 1/2, we observed a significant decrease of nauplii mortality from 100% to 30% ($p < 0.0001$; Fig. S1C); whereas significant decrease of toxicity (about 50%) has been shown ($p < 0.0001$) when the concentrations of the NAP and FLT was decreased of 1/3 and from 1/2 to 1/3 respectively,. As reported in Figs. S1D and a significant decrease of adult dead has been observed only when the NAP and FLT concentrations have been decreased of 1/2-1/3 and 1/3-1/4, respectively. The data reported at these concentrations were statistically significant as compared to control and others ratios ($p < 0.0001$).

After nauplii exposure to NAP + BkF mixture, a statistically significant increase of about 16% in toxicity was observed respect to the control ($p < 0.001$) when NAP and PHE concentration were increased by 1–4 times (Fig. S1E); whereas, an increase of the percentage of dead (ranging from 30% to 70%) with respect to control was induced only when NAP concentration was increased by 4 times and BkF concentration was increased by 4–10 times ($p < 0.0001$; Fig. S1E). However, by considering the case in which NAP amount was increased by 5–10 times and BkF concentration was increased by 2–10 times, we observed a statistically significant increase in mortality ranging from 80 to 100% respect to the control and lowest ratios ($p < 0.0001$; Fig. S1E). As reported in Fig. S1F, after adult exposure to the same mixture, we observed a statistically significant increase of about 60% in toxicity

respect to the control ($p < 0.0001$) and ratios 1:1 ($p < 0.01$) and 1:2 ($p < 0.05$) only when both concentrations have been doubled.

PHE + FLT mixture showed high toxicity both on nauplii and adults at nearly all tested ratios (Figs. S1G–H). Specifically, we observed the 100% of nauplii mortality in ratios where the concentration of the PHE was decreased from 1 to 1/4 and that of the FLT up to 1/6 ($p < 0.0001$). Considering the ratios where the concentration of the PHE was decreased from 1/5 to 1/6, significant decrease of toxicity (from 50% to 20%) has been shown ($p < 0.0001$; Fig. S1G). As reported in Figs. S1H and a significant decrease of adult dead has been observed only when the PHE and FLT concentrations have been decreased of 1/7-1/8 ($p < 0.0001$).

PHE + BkF mixture was able to induce 100% of nauplii and adult mortality in ratios where the concentrations of the PHE and BkF was decreased up to 1/6 ($p < 0.0001$; Figs. S1I–J). Only when both concentrations were decreased up to 1/6, a significant decrease of toxicity (about 40%–50%) has been observed ($p < 0.0001$; Figs. S1I–J).

After nauplii exposure to FLT + BkF mixture, a statistically significant decrease of about 25% in toxicity was observed respect to the control and ratio 1:1 ($p < 0.0001$) in ratios where FLT and BkF concentrations were decreased up to 1/2 and from 1/2 to 1/4, respectively (Fig. S1K). By considering the case in which FLT amount was only decreased up to 1/3, we observed a significant decrease of toxicity (about 20%) as compared to control ($p < 0.05$) and ratio 1:1 ($p < 0.0001$; Fig. S1K); whereas the ratios 1/3:1/3 and 1/3:1/4 showed about 10% of mortality. The data reported at these concentrations were statistically significant as compared to ratios 1:1 ($p < 0.0001$), 1:1/2 ($p < 0.001$), 1:1/3 e 1/2:1 ($p < 0.05$; Fig. S1K). As reported in Fig. S1L, after adult exposure to the same mixture, we observed a statistically significant decrease of about 60% in toxicity respect to the control ($p < 0.0001$) and ratios 1:1, 1:1/2, 1:1/3 and 1/4 ($p < 0.05$) only when both FLT concentration have been decreased from 1/2 to 1/4. By considering the case in which BkF amount was decreased up to 1/4, we observed a significant decrease of toxicity (about 25%) as compared to control ($p < 0.05$) and other ratios ($p < 0.0001$; Fig. S1L).

3.1.2. *Tertiary mixture toxicity*

NAP + PHE + FLT, NAP + PHE + BkF and PHE + FLT + BkF mixtures were able to cause the 100% of nauplii mortality ($p < 0.0001$) almost in all tested ratio (Figures S2 A,C,G). Specifically, NAP + PHE + FLT and

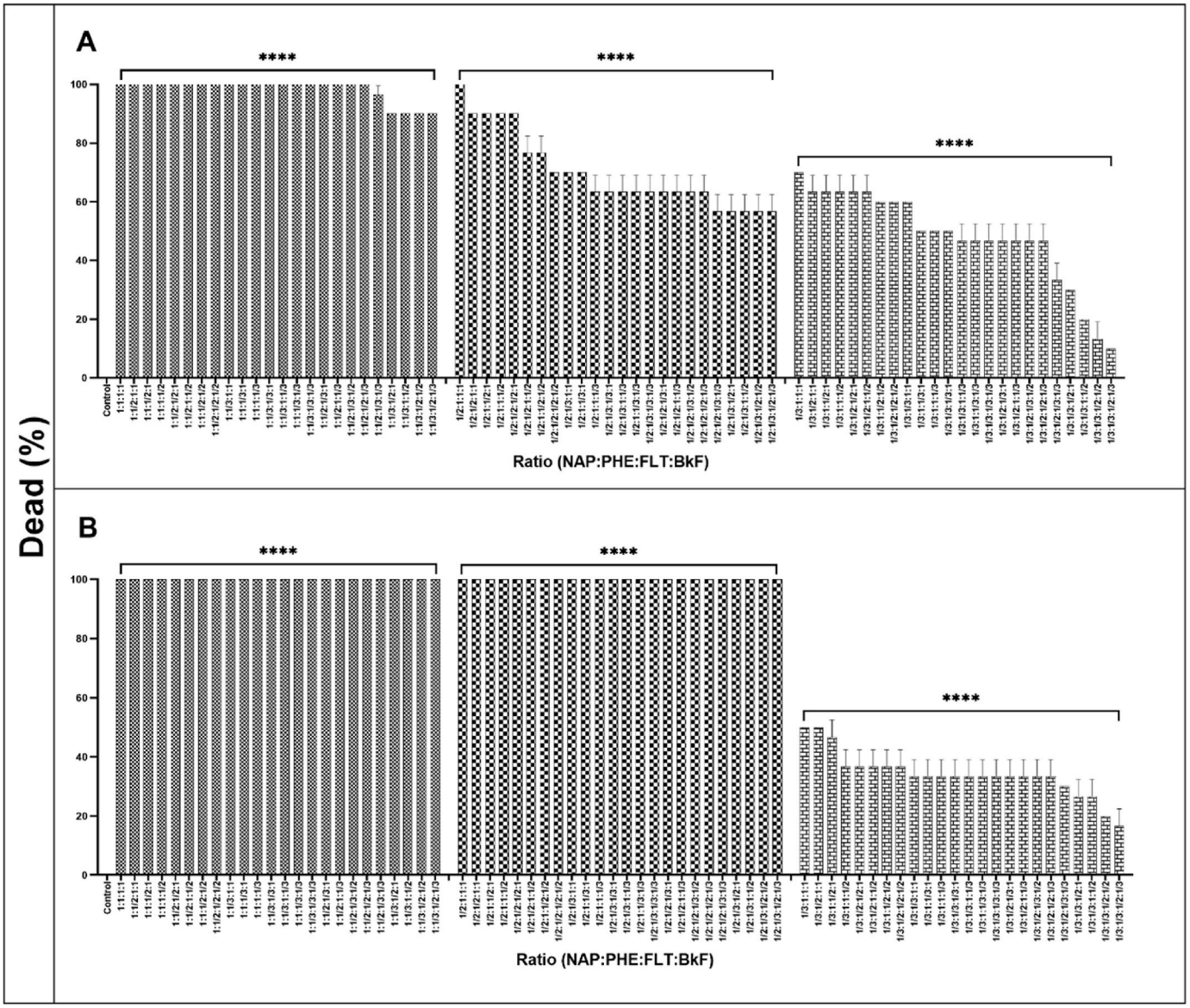

Fig. 1. The percentage of dead nauplii (A) and adults (B) after 48 h of exposure in control (0 μg/L) and treated samples with mixture of four PAHs at different ratios (Table S2) was regarded. Data are reported as mean ± standard deviation two-way ANOVA by Tukey's test (****$p < 0.0001$).

PHE + FLT + BkF mixtures caused a statistically significant decrease of about 50%–40% in toxicity when the three PAHs decreased up to 1/5 ($p < 0.0001$; Figs. S2A,C,G). By considering adults exposure to PHE + FLT + BkF mixture, a decrease of about 50%–40% in toxicity by testing the ratios 1/5:1/4:1/5 and 1/5:1/5:1/5 (Fig. S2H). As reported Figures S2B and D, a significant decrease of NAP + PHE + FLT and NAP + PHE + BkF mixtures toxicity (about 20%) on adults was regarded when the amount of PHE and FLT/BkF has been halved. These results were statistically significant as compared to the control ($p < 0.001$) and ratios 1:1:1 and 1:1/2:1 ($p < 0.0001$; Figs. S2B and D). When the concentration of NAP was also reduced to 1/2, a significant decrease in mortality (from 40% to 10%) was observed as compared to ratios 1:1:1 and 1:1/2:1 ($p < 0.0001$), 1:1:1/2 and 1:1/2:1/2 ($p < 0.01$; Figs. S2B and D).

After nauplii exposure to NAP + FLT + BkF mixture, we observed a statistically significant decrease in toxicity (about 30%–20%) as compared to control and ratios 1:1:1 ($p < 0.0001$; Fig. S2E) by considering the ratios 1:1/2:1 and 1:1:1/2. When the concentration of NAP was also reduced to 1/2, a significant decrease in mortality (10%) was observed as compared to control ($p < 0.0001$), ratios 1:1:1, 1:1/2:1 and 1:1:1/2 ($p < 0.0001$), 1:1/2:1/2 ($p < 0.01$; see also Fig. S2E). As reported Figs. S2F and a significant decrease of adult dead ($p < 0.0001$) has been observed only when the NAP, FLT and BkF concentrations have been decreased of 1/3, 1/2-1/3 and 1/2-1/3, respectively.

3.1.3. Quaternary mixture toxicity

Results of quaternary mixture toxicity experiments are given in Fig. 1. After nauplii exposure, a statistically significant decrease of about 70%–60% in mortality was observed respect to the control ($p < 0.0001$) and the lowest ratios ($p < 0.0001$) when NAP concentration was decreased by 1/2 and concentration of other PAHs was decreased by 1/2 to 1/3 (Fig. 1A).

When NAP concentration was also reduced by 1/3, a significant decrease of the percentage of dead (from 40% to 10%) with respect to control ($p < 0.01$) and other ratios ($p < 0.0001$) was induced (Fig. 1A). Finally, by considering adults exposure to NAP + PHE + FLT + BkF mixture, a decrease of toxicity was observed only when NAP concentration was decreased by 1/3 (Fig. 1B). In particular, a statistically significant decrease ranging from 50% to 16% in toxicity was observed respect to the control and the lowest ratios ($p < 0.0001$; Fig. 1B).

3.2. Gene response to PAHs mixture

As shown in Fig. 2 (see also Table S4 for the values), all binary mixture increased the expression levels of two genes (*hsp26* and *hsp60*). *hsp70* was up-regulated by NAP + BkF, PHE + FLT, PHE + BkF mixtures, and down-regulated by FLT + BkF mixture; whereas *COXI* and *COXIII* genes were up-regulated by NAP + PHE, NAP + FLT, PHE + FLT and PHE + BkF mixtures; and down-regulated by the exposure to NAP + BkF and FLT + BkF mixture (see also Supplementary Table S4).

By considering the gene expression levels of adults, common molecular target for six binary mixture was only *hsp60* gene; whereas *COXIII* was up-regulated by all binary mixture with exception of NAP + BkF that was not able to target this gene. Moreover, *hsp26* was up-regulated by NAP + BkF, PHE + FLT and FLT + BkF mixtures; and down-regulated only by PHE + BkF mixture. *hsp70* resulted down-regulated after NAP + FLT, NAP + BkF, PHE + FLT and PHE + BkF exposure, and up-regulated by FLT + BkF mixture. Finally, *COXI* was up-regulated only by FLT + BkF mixture (see Table S4 for the values).

By considering the gene expression levels of nauplii, common molecular targets for four tertiary mixtures were *hsp26, hsp60*, *COXI* and *COXIII*. In fact, these genes resulted up-regulated by all mixtures, with exception of *COXI* that was down-regulated only by NAP + PHE + BkF mixture (Fig. 2; see Table S5 for the values). *hsp70* was up-regulated by all tertiary mixtures with exception of NAP + PHE + BkF mixture that

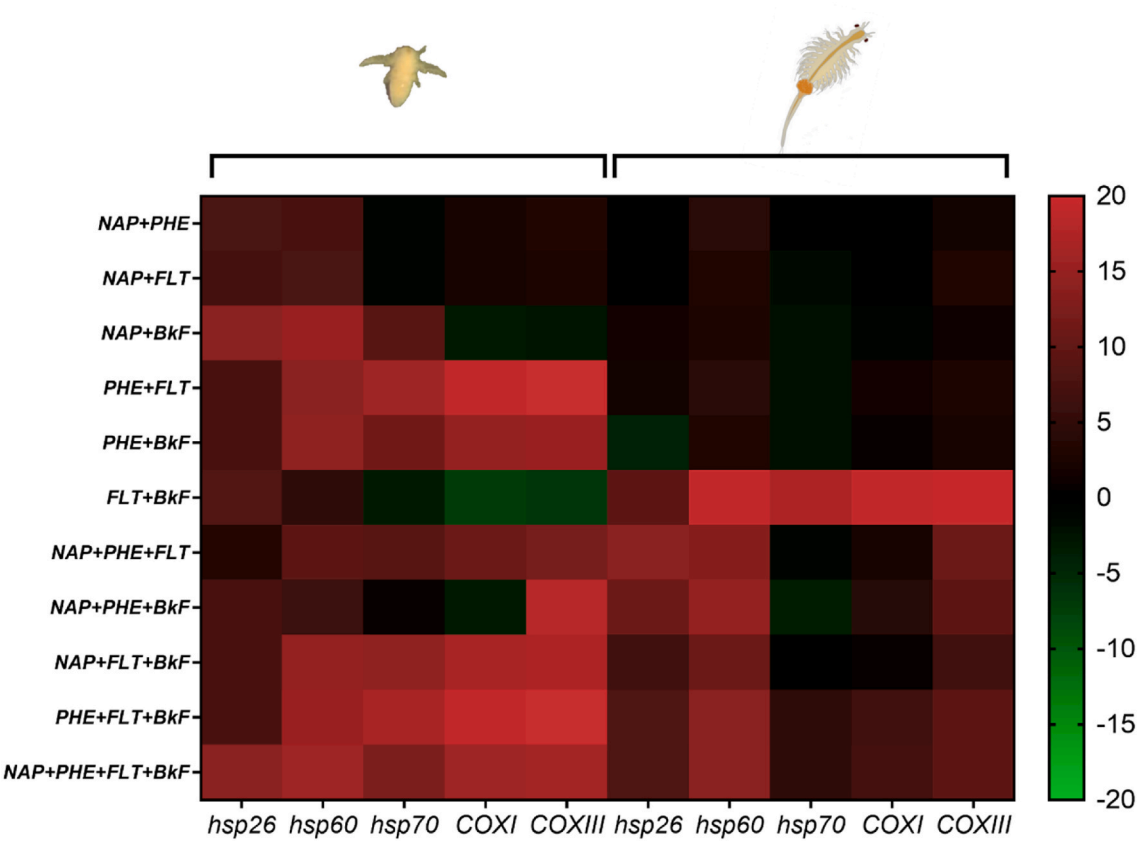

Fig. 2. Heatmaps showing the expression levels of five genes involved in stress response analyzed through real-time qPCR in *A. franciscana* nauplii and adults exposed to binary, tertiary and quaternary mixtures. Up-regulated and down-regulated genes have been represented in red and green, respectively.

was not able to target this gene (Fig. 2).

As reported in Fig. 2, common molecular targets for four tertiary mixtures were *hsp26, hsp60* and *COXIII* considering adults exposure. These genes resulted up-regulated by all mixtures (Table S5). *hsp70* gene was up-regulated and down-regulated by PHE + FLT + BkF and NAP + PHE + BkF mixtures, respectively; whereas *COXI* was up-regulated by all mixtures with exception of NAP + FLT + BkF mixture, that was not able to target this gene (Fig. 2). By considering the gene expression levels of nauplii and adults after quaternary mixture exposure, all tested genes resulted up-regulated (Fig. 2; see also Table S5).

4. Discussion

Our work explored the noxious effects of polycyclic aromatic hydrocarbon, to which marine organisms are commonly exposed in their natural habitats (Arienzo et al., 2017a; Morroni et al., 2020). When tested separately, these compounds induced about 50% dead nauplii and adults at concentrations of 0.41 and 4.23 μg/L for NAP, 7.48 and 48.7 μg/L for PHE, 0.81 μg/L for FLT and 2.4 μg/L for BkF, respectively (Albarano et al., 2022b). Interestingly, when these PAHs were mixed, the same percentage of dead nauplii and adults was observed at concentrations that were one-fourth of those used in individual tests (see Figs. S1 and S2). Only for NAP + PHE and NAP + BkF mixtures, to obtained about 50% of effect we increased the concentrations (used in individual tests) until to four/five and two times for nauplii and adults, respectively (Fig. S1).

We demonstrated for the first time that mixtures of NAP, PHE, FLT and BkF can have very strong effects on crustacean survival compared to the single compounds. In fact, all together these findings revealed that PAHs in mixtures have a predominantly synergistic and additive action, with increased harmful effects when tested together (Fig. 3). Specifically, all binary mixture showed synergic effect with exception of NAP + PHE and NAP + BkF mixtures that produced antagonism impact (CI = 22.3 and 7.8, respectively; see also Fig. 3a). As reported Fig. 3b–e, all tertiary mixtures caused synergic effect except NAP + FLT + BkF combination showing additive impact (CI = 1; Fig. 3e). At the same manner, when four PAHs were combined together, additive action have been observed (CI = 1; Fig. 3f). Thus, additive or synergistic effects could account for the developmental toxicity of crude oil even when individual PAHs are present at low concentrations (de Santana et al., 2021; Hodson, 2017; Honda and Suzuki, 2020; Meador and Nahrgang, 2019; Ramachandran et al., 2006, 2004).

In literature, toxicity increase both in aquatic vertebrates and invertebrates after PAHs combination exposure was already evidenced by several authors (Bellas et al., 2008; de Santana et al., 2021; Ikenaka et al., 2013; Patel et al., 2020). This may be due to synergistic or additive interactions at the cellular level, where compounds mutually influence how they are absorbed, metabolized, or interact with cellular receptors (Billiard et al., 2006; Hodson, 2017; Honda and Suzuki, 2020).

To date, expression profiles of genes involved in the surveillance system for chemical or physical stress, namely as the "defensome", have been used as molecular biomarkers for environmental monitoring in bioindicator species (Dalzochio et al., 2016; Marrone et al., 2012; Varrella et al., 2014). These genes are involved in detoxification processes (*i.e.* cytochrome P450; CYP), antioxidation (*i.e* glutathione S-transferase; GST), and stress response (*i.e* heat shock proteins; HSPs). In *A. franciscana*, the only identified defence genes are several HSPs (Gbotsyo et al., 2020; Han et al., 2021; Junprung et al., 2019; MacRae, 2003; Varó et al., 2019). In this study, to understand PAHs effects on *A. franciscana* cellular defence mechanisms, the transcriptional regulation of three HSPs and two COXs was examined in response to a mixture of PAHs. Almost all tested genes (with the only exception of *hsp70* at nauplii stage and, *hsp26* and *COXI* at adult stage) were up-regulated by binary, tertiary and quaternary mixture (Tables S4–S5). In general, the heat shock proteins (HSPs) are ubiquitous proteins playing a key role in an anti-water deficit system, including mitochondrial inner membrane protein, proteases, mitochondrial transmembrane protein and proteins from thermal shock (Chen et al., 2009; Wang et al., 2007). Together, by investigating the transcription profile of heat shock proteins and cytochrome oxidases, we demonstrated that the cellular defense system in *A. franciscana* may have contributed to its tolerance versus PAHs mixtures. Furthermore, the transcriptional regulation of the "defensome" in response to the different concentrations suggested that this 3 HSPs and 2 COXs could be used as molecular biomarkers for PAH-induced toxicity in *A. franciscana*.

Overall, our investigation provides further evidence that PAHs can pose a serious threat to the well-being of marine environments. In fact, when tested in mixtures, they induced a synergistic or additive effect, suggesting that their impact could be probably higher than those reported in previous studies testing individual compounds (Bellas et al., 2008; Honda and Suzuki, 2020; Albarano et al., 2022b). This is quite alarming, since marine organisms are exposed to several organic

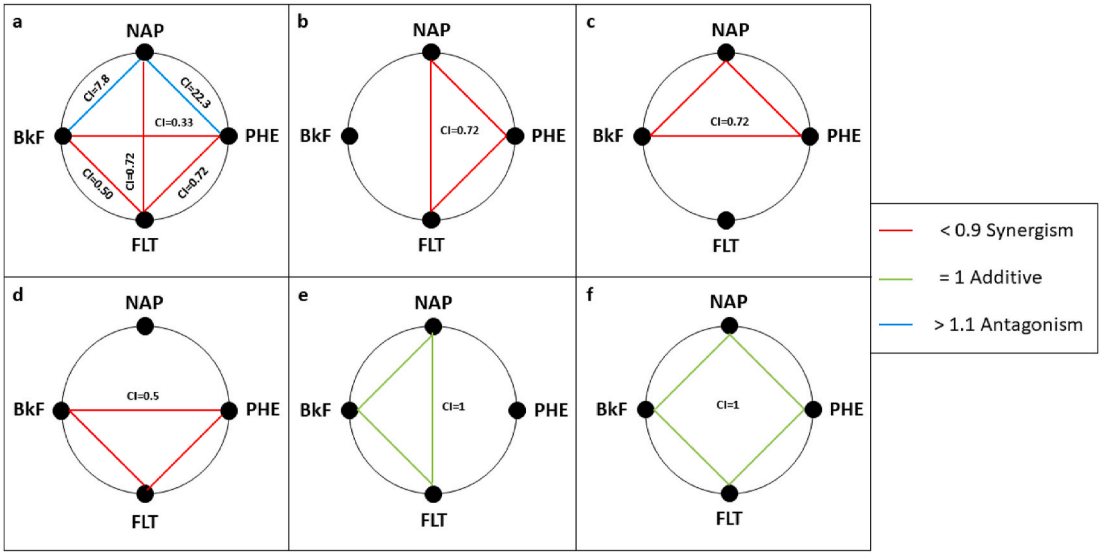

Fig. 3. Polygonogram for 4 PAHs. naphthalene (NAP), phenanthrene (PHE), fluoranthene (FLT), and benzo-k-fluoranthene (BkF), were combined in (a) binary, (b–e) tertiary and (f) quaternary mixtures. Red, green and blue lines indicate respectively synergism, additive and antagonism between the investigated organic compounds.

compounds at the same time through dietary, sediment and water exposure. Once they are uptaken, PAHs can be easily accumulated in aquatic organisms due to their high lipophilicity (Albarano et al., 2021a; Wan et al., 2007). It has been widely demonstrated that PAHs are more accumulated in invertebrates (Albarano et al., 2021a; Baumard et al., 1998; Li et al., 2021; Palmqvist et al., 2006; Ruocco et al., 2020). For these reasons, marine invertebrates are abundantly used in understanding the toxicological effects due to high levels of organic compounds concentrations (Gambardella et al., 2017; Kuntke et al., 2020; Ojija and Laizer, 2016; Pagano et al., 2017).

5. Conclusion

Naphthalene, phenanthrene, fluoranthene and benzo-k-fluoranthene as mixtures can increase the adverse effects on *A. franciscana*. Specifically, i) almost all binary (NAP + FLT (CI = 0.72), BkF + FLT (CI = 0.50), PHE + FLT (CI = 0.72), PHE + BkF (CI = 0.33)) and tertiary (NAP + PHE + FLT (CI = 0.72), NAP + PHE + BkF (CI = 0.72) and PHE + FLT + BkF (CI = 0.50)) mixtures showed synergism; ii) only NAP + PHE (CI = 22.3) and NAP + BkF (CI = 7.8) mixtures produced antagonism; iii) quaternary NAP + FLT + BkF and NAP + PHE + FLT + BkF (CI = 1) displayed additive action; and iv) all tested combinations induced variations in the expression of genes, involved in stress response in both nauplii and adult of *A. franciscana*. These results are of a significant ecological relevance and stimulate further testing of PAHs mixtures to better explore how their interactions may affect the biological processes of marine invertebrates.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122286.

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