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Article

Genotoxicity Set Up in *Artemia franciscana* Nauplii and Adults Exposed to Phenanthrene, Naphthalene, Fluoranthene, and Benzo(k)fluoranthene

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Abstract: Polycyclic aromatic hydrocarbons (PAHs) consist of a group of over 100 different organic compounds mainly generated by anthropogenic activities. Because of their low water solubility, they tend to be accumulated in sediment, where their degradation rate is very low. Few studies have been carried out so far to investigate the effects of PAHs on *Artemia franciscana*. *Artemia* is easy to manage at laboratory scale, but it is not a sensitive biological model considering the traditional endpoints (i.e., mortality). In addition to evaluating the lethality on nauplii and adults of *A. franciscana* after 24 and 48 h, we focused on the genotoxicity to investigate the potential effects of phenanthrene (PHE), naphthalene (NAP), fluoranthene (FLT), and benzo(k)fluoranthene (BkF). Results showed that FLT was the most toxic both for nauplii and adults after 48 h of exposure. Real-time qPCR showed that all toxicants, including BkF, which had no negative effects on the survival of the crustacean, were able to switch the gene expression of all nine genes. This work has important ecological implications, especially on contaminated sediment assessment considering that PAHs represent the most abundant organic group of compounds in marine environment, opening new perspectives in understanding the molecular pathways activated by crustaceans.

Keywords: polycyclic aromatic hydrocarbons; crustacean; short-term effects; toxicity; genotoxicity

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of over 100 different organic compounds generated by natural events or anthropogenic activities. PAHs predominantly originate from anthropogenic processes, especially from incomplete combustions of organic fuels. Certain naturally occurring processes, such as volcanic eruptions and forest fires, contribute to the increase of these organic compounds in the environment. In Santos Bay and Estuary, the anthropogenic contributions to PAHs in sediments resulted of about 99% (i.e., concentrations varied from 79.6 for uninhabited area to 15,389.1 ng/g for area located in the proximity of industries [1]).

PAHs are formed by two or more fused benzene rings, and their toxicity depends on the number of benzene rings [2–4]. Because of their low water solubility and hydrophobicity, in the water column, PAHs tend to associate with suspended particulate matter and are

eventually deposited in sediments, where their degradation is very slow [5]. The level of PAHs in the water column is closely linked to the level of PAHs in sediments [6,7]. In fact, PAHs concentration in the water column increase with increased concentration in surface sediment [6,7]. Although they are not very soluble in water, their concentration in the water column remains stable for a long time, thus representing a great problem for biota and consequently for human health [8–10]. Specifically, the half-lives of low-molecular-weight PAHs (naphthalene, acenaphthene, fluorene, and phenanthrene) ranged from approximately 3 to 8 days, whereas half-lives of high-molecular-weight PAHs (pyrene, chrysene, benzo[a]pyrene, dibenz[a,h]anthracene) ranged from 73 to 1780 days [11–13].

For these reasons, the European Water Framework Directive 2000/60/EC (WFD) was developed aiming to achieve and ensure good ecological and chemical water status [14]. The list of monitored pollutants has recently been updated with a new daughter Directive (2013/39/EU) to identify a number of emerging chemicals of concern, including non-polar organic substances (e.g., PAHs and PCB) and polar compounds (e.g., pharmaceuticals and pesticides) [15].

In aquatic environments, PAHs can have several toxic effects, such as immunotoxicity, embryotoxicity, and cardiotoxicity, especially impacting fish, benthic organisms, and other marine vertebrates [16–19]. Five different PAHs (naphthalene (NAP), phenanthrene (PHE), fluoranthene (FLT), fluorene (FLR), pyrene (PYR), and hydroxypyrene), known to be potentially toxic, inhibited and reduced the larval development and growth of both *Mytilus galloprovincialis* and *Paracentrotus lividus*, whereas NAP was able to impact the embryos and larval stages of *Ciona intestinalis* [16]. The benzo(a)anthracene (BaA), one of the most toxic PAHs, showed higher toxicity on crustaceans *Daphnia magna* (LC50 = 4.3 µg/L) and *Ceriodaphnia reticulata* (LC50 = 4.7 µg/L) than that displayed *Artemia salina* at conceivable concentrations in the environment (from 1 to 32 µg/L) [20,21]. At the same manner, PHE and FLT were able to impact the survival of *D. magna* (LC50 = 50 and 10 mg/kg, respectively), *Hyalella azteca* (LC50 = 15 and 5 mg/kg, respectively), and *Chironomus riparius* (LC50 = 20 and 15 mg/kg, respectively) [22]. After PYR, FLT, and anthracene (ANT) exposure, *D. magna* and *Artemia salina* crustaceans displayed higher sensibility than those registered for the mosquito *Aedes aegypti*, the amphibian *Rana pipiens*, and the fish *Pimephales promelas* [23].

To the best of our knowledge, few studies have been carried out so far to investigate direct toxic effects of individual PAHs on *A. franciscana*, but no work has been conducted to establish the possible changes in expression levels of genes after organic compounds exposure. Rojo-Nieto et al. [24] established that mixtures of ten PAHs (naphthalene, acenaphthene, phenanthrene, fluoranthene, fluorene, pyrene, anthracene, benzo(a)pyrene, benzo(a)anthracene, and chrysene) found in sediment samples from the Bay of Algeciras did not have impact on survival of *A. franciscana* using passive dosing. Similarly, the passive dosing of three PAHs (toluene, 1-methylnaphthalene, and phenanthrene) did not impact the hatching of cysts [25].

The crustacean *A. franciscana* has been considered as a model species to investigate the ecotoxicological response of marine invertebrates to environmental pollutants [26–28]. The main advantage of this species is that nauplii can be hatched as needed from commercially available durable cysts to avoid the maintenance of laboratory cultures as required for many model species used in ecotoxicity tests. In any case, these tests (namely “Toxkit”) employing dormant stages (“cryptobiotic eggs”) have the same efficacy and sensitivity as tests with cultured animals [29]. Moreover, the embryo hatches and grows rapidly in laboratory conditions (the nauplius stage is reached in 24 h), and the small body size permits to conduct tests in small beakers or even plates. In addition, *Artemia* is a eurialine organism with large adaptability to a range of salinities (5–300 PSU) and temperatures (6–40 °C) [30]. However, *Artemia* models revealed several disadvantages due to a limited sensitivity towards a wide range of substances in comparison to other species so that the possibility to underestimate potential effects may occur [26,31]. In fact, in the recent years, the use of this crustacean in ecotoxicology has become increasingly rare [31]. For these reasons, in this work, we are interested in giving a new life to this model organism by proposing

genotoxicity as a new endpoint. Since changes of gene expression induced by some toxicants may be very subtle and differences of animal reactivity between experimental groups may not be noticed by simple observations, the genotoxicity could be considered a good approach providing more detailed toxicological information. Therefore, the use of *A. franciscana* for evaluating the molecular aspects that are on the base of toxicological effects could confirm this branchiopod crustacean as a good biological model.

Thus far, few studies investigated the stress response of *Artemia* spp. through the evaluation of key genes involved in larval growth, molting, stress, and detoxification processes [32–37]. In this work, as well as evaluating PHE, NAP, FLT, and BkF acute (24 h–48 h-LC50) toxicity on nauplii and adults by measuring survival, we defined for the first time the molecular response of PAHs toxicity. In particular, after 48 h under sublethal exposure for both tested life stages, the effect on several key genes involved in stress response (*hsp26*, *hsp60*, *hsp70*, *COXI*, and *COXIII*) was assessed. In addition, the impact on developmental genes (*HAD-like*, *tcp*, *UCP2*, and *CDC48*) was also evaluated for nauplii.

Sediment can be the final main sink and source of PAHs and genotoxicity can represent an easy and fast screening method for their ranking [38–40]. Prior to direct PAHs contaminated sediment investigation, we decided to highlight the sensitivity of genotoxicity endpoint in *A. franciscana* from spiked saltwater solutions.

In this study, we tested the PHE, NAP, FLT, and benzo(k)fluoranthene (BkF) toxicity on embryos and adults of the branchiopod crustacean *A. franciscana* Kellog 1906, using environmental concentrations (from 0.025 to 10 mg/L, from 0.36 to 2.3×10^2 mg/L, from 0.41 to 3.9×10^2 mg/L, and from 0.025 to 9.4×10^1 mg/L for NAP, PHE, FLT, and BkF, respectively) detected in polluted sediments subjected to various pollution sources [38].

2. Materials and Methods

2.1. Ecotoxicity Test

Acute toxicity test using both *A. franciscana* nauplii and adults were performed according to standard methods [41] using lethality as an endpoint. Effects were measured after 24 and 48 h of exposure for both adults and nauplii up to the third instar (corresponding to 48 h old specimen that are considered as the most sensitive stage). Certified dehydrated cysts of brine shrimp *A. franciscana* (AF/F2005) were purchased from the company ECOTOX LDS (Gallarate, Italy). Hatching of the cysts was obtained by incubating 100 mg of cysts in glass Petri dishes containing seawater prepared by dissolving 36 g of Instant Ocean[®] salt in deionized water, stirred for 24 h under aeration and then filtered through 0.45 μm Millipore cellulose filters. Newly hatched brine shrimp larvae (Instar I nauplius stage) were separated from unhatched cysts and transferred, taking advantage of phototactic movements, into new glass Petri dishes with synthetic seawater (SSW) prepared according to ISO 10253/16 [42].

2.2. Chemicals

The naphthalene, phenanthrene, fluoranthene, and benzo(k)fluoranthene (Sigma-Aldrich, Saint Louis, USA) were used in the toxicity tests. The purity was greater than 97%. Stock solutions of NAP, PHE, FLT, and BkF were prepared by dissolving the above indicated chemicals in dimethyl sulfoxide (DMSO) [43–45]. Maximum DMSO in test solutions did not exceed 1% *v/v*, which is not toxic to *A. franciscana* [46]. The solubility of PAHs in seawater depends on temperature, salinity, and the analytical method used for the determination. Details about were reported in Supplementary Materials (Supplementary Table S1). Autoclaved glass tubes containing stock solutions (XX mg/L) of each PAH in DMSO were kept in the dark at room temperature. The solutions were sampled and analyzed for four PAHs according to Carotenuto et al. [47].

2.3. Acute Toxicity Test

Acute toxicity test on nauplii was performed by adding 10 nauplii to each well of 24-well plates, containing 2 mL of solutions at increasing concentrations of NAP

(0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.5, 1, 2.5, 5, 10 mg/L), PHE (0, 0.36, 1, 2, 3, 4, 5, 10, 57.5, 115, 230 mg/L), FLT (0, 0.41, 1, 2.5, 5, 12.5, 25, 50, 97.5, 195, 390 mg/L), and BkF (0, 0.025, 0.5, 1, 1.5, 3, 6, 12, 23.5, 47, 94 mg/L) tested in SSW.

Toxicity on adults was evaluated by adding 5 crustaceans to each well of 6-well plates, containing 10 mL with solutions of various concentrations of NAP, PHE, FLT, and BkF mentioned above. The plates were kept at 25 ± 1 °C with salinity 35 ppm for 48 h in a light regime of 16:8 h light:dark, without providing food. At 24 and 48 h, the number of nauplii and adults (which were motionless for 10 s) was counted under a stereomicroscope (Leica EZ4 HD) to calculate the mortality. Tests were considered as valid when mortality in control (organisms exposed to 0 mg/L of organic compounds) was <10% after 48 h. All the experiments were performed in triplicates.

2.4. Organisms Exposures for RNA Extraction

Two hundred nauplii of *A. franciscana* were exposed to NAP, PHE, FLT, and BkF at 0.26 mg/L, 1.15 mg/L, 0.81 mg/L, and 84.6 mg/L, respectively, whereas 10 *A. franciscana* adults were exposed to NAP, PHE, FLT, and BkF at 1.45 mg/L, 1.15 mg/L, 0.81 mg/L, and 84.6 mg/L, respectively. These concentrations were chosen because they did not cause mortality in acute tests. All the experiments were performed in triplicates.

Samples were collected after 48 h of exposure by centrifugation at $4000 \times g$ for 15 min in a swing-out rotor at 4 °C in a 2 mL tube, kept on ice, and were further homogenized in TRIzol (Invitrogen, Paisley, UK) using a TissueLyser II (Qiagen, Valencia, CA, USA) and steel beads of 7 mm diameter (Qiagen, Valencia, CA, USA). Total RNA was extracted and purified using Direct-zol™ RNA Miniprep Plus Kit (ZYMO RESEARCH). The amount of total RNA extracted was estimated by the absorbance at 260 nm and the purity by 260/280 and 260/230 nm ratios, using a NanoDrop spectrophotometer 2000 (Thermo Scientific Inc., Waltham, MA, USA), to exclude the presence of proteins, phenol, and other contaminants [48].

2.5. cDNA Synthesis and Real Time q-PCR

For each sample, 1000 ng of total RNA was retrotranscribed with an iScript™ cDNA Synthesis kit (Bio-Rad, Milan, Italy), following the manufacturer's instructions. The variations in the expression of five genes involved in stress response (*hsp26*, *hsp60*, *hsp70*, *COXI*, and *COXIII* [49]; see Supplementary Figure S1) were evaluated for adults. For nauplii, the variations in the expression of four other genes involved in developmental and differentiation processes (*HAD-like*, *tcp*, *UCP2*, and *CDC48*, [49]) were also tested (Supplementary Figure S1).

Undiluted cDNA was used as a template in a reaction containing a final concentration of 0.3 mM for each primer and $1 \times$ SensiFAST™ SYBR Green master mix (total volume of 10 µL) (Meridiana Biotec). PCR amplifications were performed in AriaMx Real-Time PCR instrument (Agilent Technologies, Inc., Santa Clara, CA, USA), according to the manufacturer's instructions, using the following thermal profile: 95 °C for 10 min, one cycle for cDNA denaturation; 95 °C for 15 s and 60 °C for 1 min, 40 cycles for amplification; 95 °C for 15 s, one cycle for final elongation; and one cycle for melting curve analysis (from 60 °C to 95 °C) to verify the presence of a single product. Each assay included a no-template control for each primer pair. To capture intra-assay variability, all real-time qPCR reactions were carried out in triplicate. Fluorescence was measured using Agilent Aria 1.7 software (Agilent Technologies, Inc.). The relative expression ratios were calculated according to [50,51] using REST software (Version No., Relative Expression Software Tool, Weihenstephan, Germany; Equation (1)):

$$R = \frac{E_{target}^{\Delta Cq_{target}} (Mean\ Control - Mean\ Sample)}{E_{reference}^{\Delta Cq_{reference}} (Mean\ Control - Mean\ Sample)} \quad (1)$$

The expression of each gene was analyzed and internally normalized against *GAPDH* [49] using REST software (Relative Expression Software Tool, Weihenstephan, Germany) based on the Pfaffl method [50,51]. Relative expression ratios above 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 8.02 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com, accessed on 1 February 2021). Fold-change values were represented through a Heatmap generated by GraphPad Prism Software.

2.6. LC50 Calculation and Statistical Analyses

Toxicity data were reported as mean \pm standard deviation (SD). Data were checked for normality using the Shapiro–Wilk’s (S–W) test (p -value < 0.05). The significance of differences among treatments and the control was checked by two-way ANOVA followed by post hoc Tukey’s test for multiple comparisons (GraphPad Prism Software version 8.02 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com, accessed on 1 February 2021). p -Values < 0.05 were considered statistically significant. The calculation of LC50 values was done by GraphPad Software through four parameters of the logistic equation, which corresponds to the dose-response curve with the slope of the variable slope.

3. Results

3.1. PAHs Analysis

Samples were analyzed to verify NAP, PHE, FLT, and BkF nominal concentrations ranging from 0.025 to 10 mg/L, 0.36 to 2.3×10^2 mg/L, 0.41 to 3.9×10^2 mg/L, and 0.025 to 94 mg/L, respectively (shown in Table 1). The gas chromatography-mass spectrometry (GC-MS) (2010plus-TQ8030, Shimadzu, Japan) determinations showed a good agreement between nominal vs. analytical concentrations, whose ratios were less than 1.5 in most cases (Table 1).

3.2. Naphthalene, Phenanthrene, Fluoranthene, and Benzo(k)fluoranthene Toxicity on Nauplii

After 24 h of exposure to NAP, a statistically significant increase in toxicity ($p < 0.0001$; see Supplementary Table S2) was observed only at the two highest tested concentrations of 4.23 mg/L (38 % mortality) and 10.1 mg/L (43% mortality) (Figure 1A).

After 48 h, mortality became statistically significant ($p < 0.01$) already at 0.11 and 0.26 mg/L and reached 80% ($p < 0.0001$) at 4.23 and 10.1 mg/L (Figure 1A and Supplementary Table S2).

After 24 h of exposure (Figure 1B) PHE induced an increase of the percentage of dead (about 26%) with respect to control already from 3.45, 4.23, and 7.48 mg/L. The data reported at these concentrations were statistically significant as compared to the two lowest (0 and 0.21 mg/L; $p < 0.01$) and highest (98.6 and 223.4 mg/L; $p < 0.01$; see Supplementary Table S2) concentrations. At 48.7, 98.6, and 223.4 mg/L, significant increase of toxicity (about 43.3%, 50%, and 50%, respectively) respect lower tested concentrations (0, 0.21, 0.71, 1.15, and 2.26 mg/L; $p < 0.0001$; see also Supplementary Table S2) has been shown. The scenario after 48 h of exposure was similar to the one described after 24 h. About 26% of dead nauplii was registered at 1.15 mg/L ($p < 0.05$; Supplementary Table S2). At 2.26 and 3.45 mg/L, the toxicity (about 34%) was statistically significant compared to 0, 0.21, 7.48, 48.7, 98.6, and 223.4 mg/L ($p < 0.0001$; Supplementary Table S2). At 7.48, 48.7, and 98.6 mg/L, about 70% of mortality was registered, whereas a percentage of about 90% of dead was displayed at 223.4 mg/L.

Table 1. Comparisons of nominal vs. analytical concentrations of naphthalene (NAP), phenanthrene (PHE), fluoranthene (FLT), and benzo(k)fluoranthene (BkF) in seawater. The data were reported in mg/L.

Compounds	Nominal Concentration	Analytical Concentration	Nominal/Analytical Concentration Ratio
NAP	0.025	0.015	1.67
	0.05	0.032	1.56
	0.1	0.078	1.28
	0.2	0.11	1.82
	0.4	0.26	1.54
	0.5	0.41	1.22
	1	0.76	1.32
	2.5	1.45	1.72
	5	4.23	1.18
	10	10.1	0.99
PHE	0.36	0.21	1.71
	1	0.71	1.41
	2	1.15	1.74
	3	2.26	1.33
	4	3.45	1.16
	5	4.23	1.18
	10	7.48	1.34
	57.5	48.7	1.18
	115	98.6	1.17
	230	223.4	1.03
FLT	0.41	0.29	1.41
	1	0.81	1.23
	2.5	2.14	1.17
	5	4.41	1.13
	12.5	9.91	1.26
	25	20.4	1.23
	50	45.6	1.10
	97.5	91.6	1.06
	195	179	1.09
	390	325	1.20
BkF	0.025	0.016	1.56
	0.5	0.41	1.22
	1	0.78	1.28
	1.5	0.98	1.53
	3	2.4	1.25
	6	5.3	1.13
	12	10.4	1.15
	23.5	19.5	1.21
	47	41.7	1.13
	94	84.6	1.11

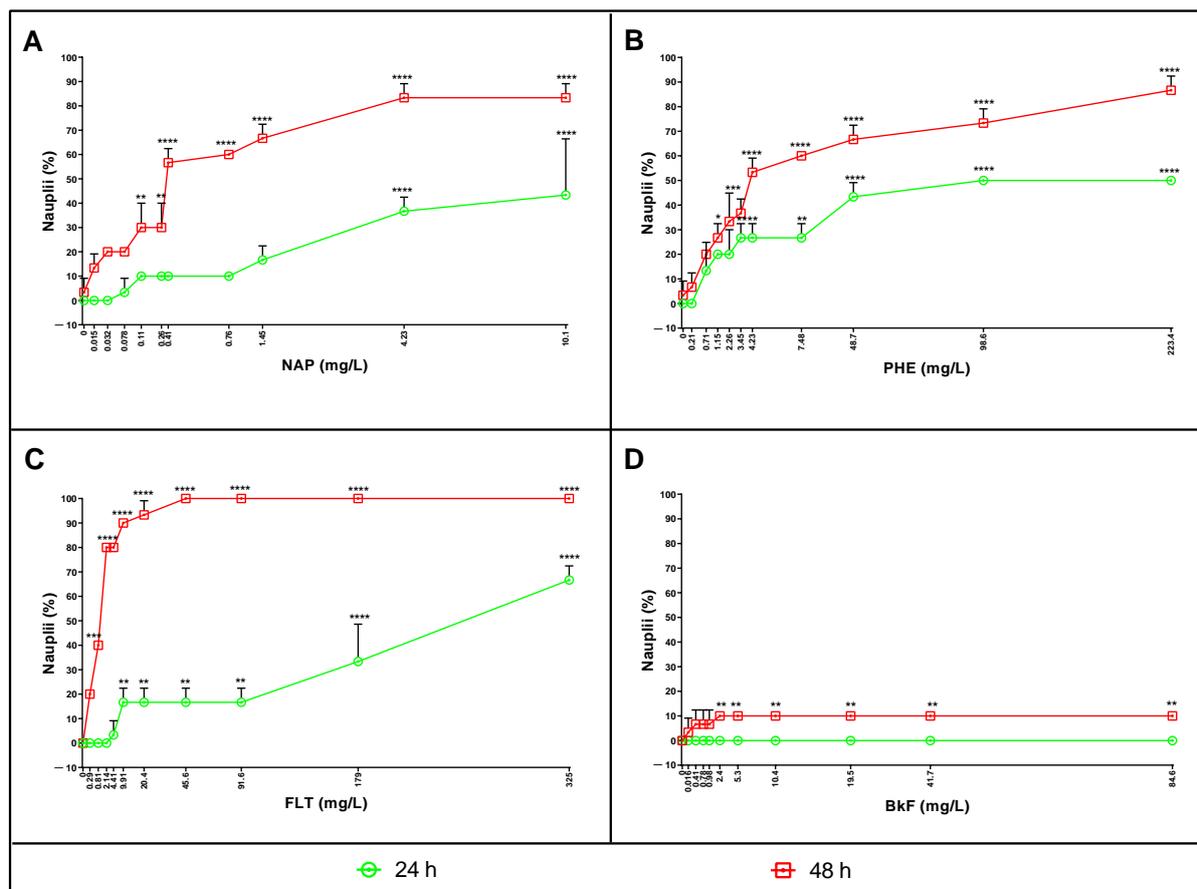


Figure 1. After 24 h and 48 h, the percentage of dead nauplii in control (0 mg/L) and treated samples with (A) NAP at the concentrations of 0.025, 0.05, 0.1, 0.2, 0.4, 0.5, 1, 2.5, 5, and 10 mg/L; (B) PHE at the concentrations of 0.36, 1, 2, 3, 4, 5, 10, 57.5, 115, and 230 mg/L; (C) FLT at the concentrations of 0.41, 1, 2.5, 5, 12.5, 25, 50, 97.5, 195, and 390 mg/L; and (D) BkF at the concentrations of 0.025, 0.5, 1, 1.5, 3, 6, 12, 23.5, 47, and 94 mg/L was regarded. Data are reported as mean \pm standard deviation two-way ANOVA by Tukey's test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Taking into the consideration FLT exposure (Figure 1C), an increase of the percentage of dead (about 18%) with respect to control was already recorded from 9.91, 20.4, 45.6, and 91.6 mg/L. The data reported at these concentrations were statistically significant compared to the four lowest (0, 0.29, 0.81, and 2.14 mg/L; $p < 0.01$) and the two highest (179 and 325 mg/L; $p < 0.0001$; see Supplementary Table S2) concentrations. At 179 and 325 mg/L, significant increase of toxicity (about 35% and 70%, respectively) with respect to lower tested concentrations (4.41, 9.91, 20.4, and 45.6 mg/L; $p < 0.0001$; Supplementary Table S2) has been shown. After 48 h, at 0.81 mg/L, a significant increase of toxicity (about 40%) has been displayed with respect to 0 ($p < 0.0001$) and 0.29 mg/L ($p < 0.001$; see also Supplementary Table S2). However, the toxicity increases of about 80% (2.14 and 4.41 mg/L). These data were statistically significant respect to compared to the three lowest (0, 0.29, and 0.81 mg/L; $p < 0.0001$) and the highest (20.4, 45.6, 91.6, 179, and 325 mg/L; $p < 0.001$; Supplementary Table S2).

When we considered BkF toxicity (Figure 1D) after 24 h, no effect has been recorded. Only after 48 h, at 2.4, 5.3, 10.4, 19.5, 41.7, and 84.6 mg/L, a significant increase in toxicity (about 10%) was shown with respect to the control ($p < 0.01$; Supplementary Table S2).

3.3. Naphthalene, Phenanthrene, Fluoranthene, and Benzo(k)fluoranthene Toxicity on Adults

After 24 h of exposure, NAP and PHE and BkF did not affect the survival of *A. franciscana* at all tested concentrations (Figure 2).

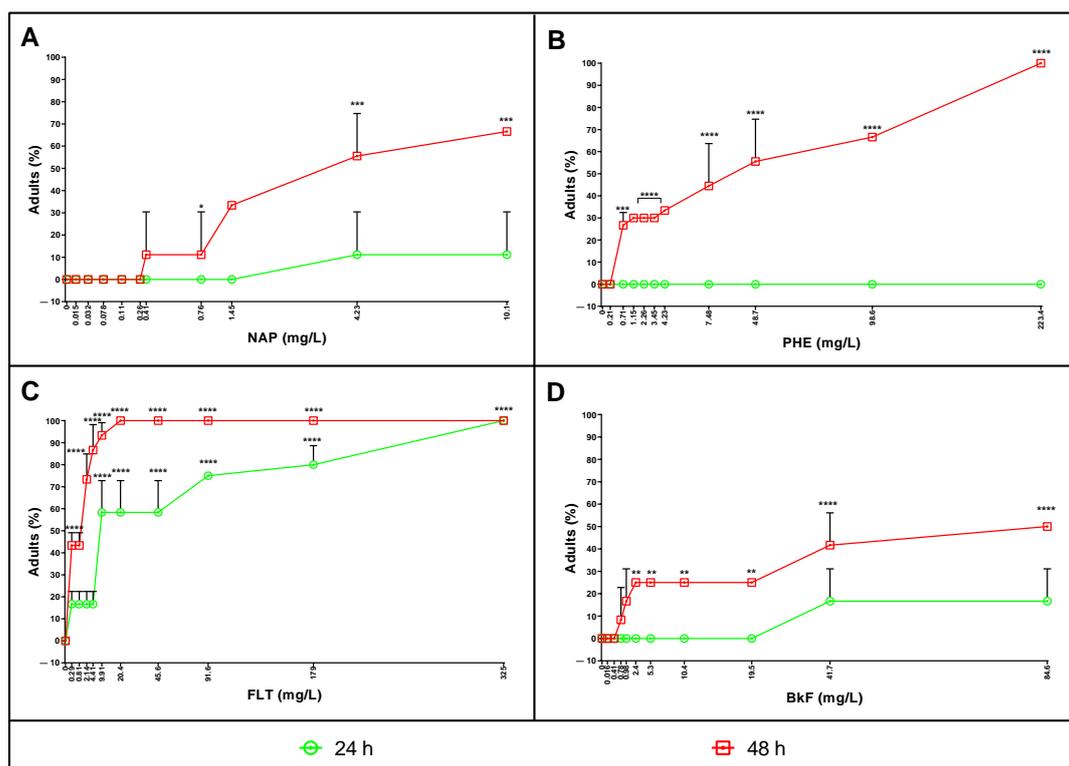


Figure 2. After 24 h and 48 h, the percentage of dead adults in control (0 mg/L) and treated samples with (A) NAP at the concentrations of 0.025, 0.05, 0.1, 0.2, 0.4, 0.5, 1, 2.5, 5, and 10 mg/L; (B) PHE at the concentrations of 0.36, 1, 2, 3, 4, 5, 10, 57.5, 115, and 230 mg/L; (C) FLT at the concentrations of 0.41, 1, 2.5, 5, 12.5, 25, 50, 97.5, 195, and 390 mg/L; and (D) BkF at the concentrations of 0.025, 0.5, 1, 1.5, 3, 6, 12, 23.5, 47, and 94 mg/L was regarded. Data are reported as mean \pm standard deviation two-way ANOVA by Tukey's test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

However, only FLT showed toxic effects already after 24 h of exposure (Figure 2C). An increase of the percentage of dead (about 60%) with respect to control was already recorded from 9.91, 20.4, 45.6, and 91.6 mg/L. The data reported at these concentrations were statistically significant compared to the four lowest (0, 0.29, 0.81, and 2.14 mg/L; $p < 0.0001$; Supplementary Table S3) and the two highest (179 and 325 mg/L; $p < 0.0001$; Supplementary Table S3) concentrations. At 179 and 325 mg/L, significant increase of toxicity (about 95% and 100%, respectively) with respect to all other concentrations ($p < 0.0001$; Supplementary Table S3) was shown. After 48 h, at 0.29 and 0.81 mg/L, a significant increase of toxicity (about 45%) was displayed with respect to 0 ($p < 0.0001$). However, toxicity increases of about 66% (2.14 and 4.41 mg/L) were shown. These data were statistically significant compared to the three lowest (0, 0.29 and 0.81 mg/L; $p < 0.0001$) and the highest (20.4, 45.6, 91.6, 179, and 325 mg/L; $p < 0.05$; Supplementary Table S3), where a percentage of about 100% was registered.

After 48 h, NAP caused a statistically significant mortality starting from 1.45 mg/L (30%), with a maximum effect of 60% at 10.1 mg/L (Figure 2A).

As shown in Figure 2B, PHE induced an increase of the percentage of dead (about 26.6%) with respect to control already from 0.71 mg/L. The data reported at this concentration were statistically significant compared to the two lowest (0 and 0.21 mg/L; $p < 0.001$) and other concentrations ($p < 0.0001$; Supplementary Table S3). At 1.15, 2.26, 3.45, 4.23, and 7.48 mg/L, significant increase of toxicity (about 30%, 30%, 30%, 33%, and 45%, respectively) with respect to lower (0, 0.21, and 0.71 mg/L; $p < 0.0001$) and higher (48.7, 98.6, and 223.4 mg/L; $p < 0.0001$; see also Supplementary Table S3) tested concentrations was shown. At 48.7 and 98.6 mg/L, the toxicity (about 66%) was statistically significant compared to

other used concentrations ($p < 0.0001$). At 223.4 mg/L, 100% of mortality was registered ($p < 0.0001$; Supplementary Table S3).

When we considered BkF toxicity (Figure 2D) after 24 h, no effect was recorded. Only after 48 h, at 2.4, 5.3, 10.4, and 19.5 mg/L, a significant increase in toxicity (about 25%) was shown with respect to the three lowest concentrations (0, 0.016, 0.41 mg/L; $p < 0.01$) and the highest concentration (84.6 mg/L; $p < 0.01$; Supplementary Table S3), where a percentage of about 50% was registered.

3.4. Lethal Concentrations after 24 and 48 h of Exposure

Considering nauplii exposure, the NAP solution has a LC50 value of 1.73 mg/L (1.52–46.28 mg/L) and 0.60 mg/L (0.21–90.38 mg/L) after 24 h and 48 h, respectively; PHE solution has a LC50 value of 4.44 (3.66–56.76 mg/L) and 3.07 mg/L (1.32–81.01 mg/L) after 24 h and 48 h, respectively; FLT solution has a LC50 value of 1.30 (0.45–107.5 mg/L) and 0.09 mg/L (0.01–99.1 mg/L) after 24 h and 48 h, respectively (Supplementary Table S4). When considering adults, the NAP solution has a LC50 value of 0.11 mg/L (0.02–12.06 mg/L) and 44.31 mg/L (5.81–268.12 mg/L) after 24 h and 48 h, respectively; PHE solution has a LC50 value of 1.68 mg/L (1.35–234.09 mg/L) after 48 h; FLT solution has a LC50 value of 32.03 mg/L (0.10–120.08 mg/L) and 0.77 mg/L (0.10–103.67 mg/L) after 24 h and 48 h, respectively; and BkF solution has a LC50 value of 28.67 mg/L (0.5–36.67 mg/L) and 6.12 mg/L (0.05–48.72 mg/L) after 24 h and 48 h, respectively (see also Supplementary Table S4).

3.5. Gene Response to NAP, PHE, FLT, and BkF Exposure

Five genes were analyzed for adults, and all were targeted by four PAHs with the exception of *hsp70*, *COXI*, and *COXIII* (Figure 3; see also Supplementary Table S5 for the values).

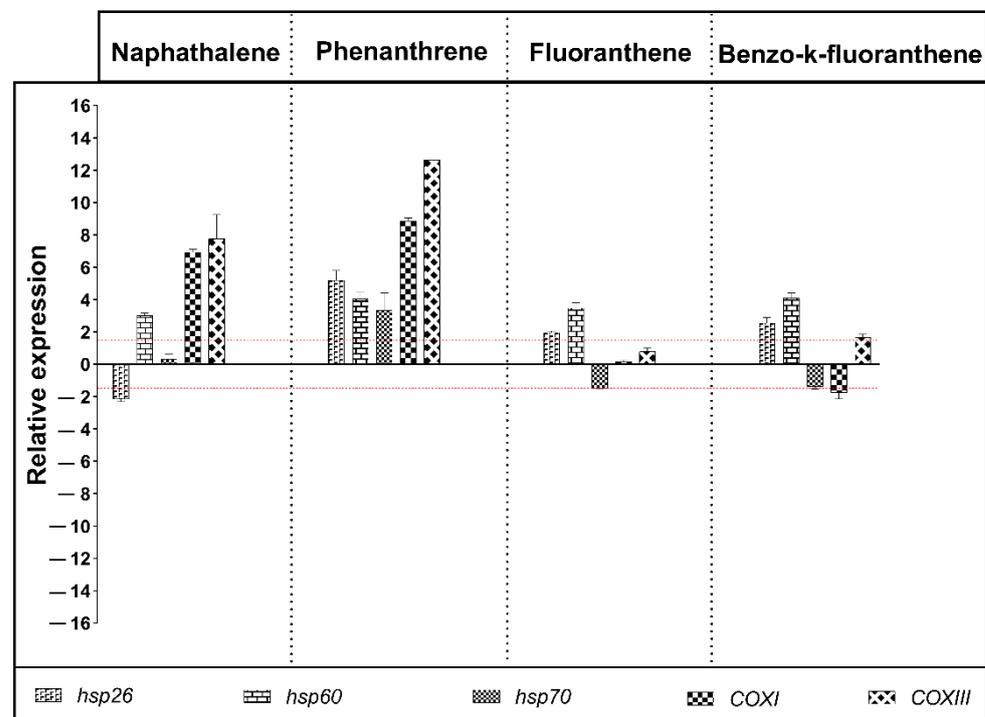


Figure 3. Histograms show the differences in expression levels of five genes involved in stress response. *A. franciscana* adults were exposed to naphthalene, phenanthrene, fluoranthene, and benzo(k)fluoranthene at 1.45 mg/L, 1.15 mg/L, 0.81 mg/L, and 84.6 mg/L, respectively. Fold differences greater than ± 1.5 (see red dotted horizontal guidelines at values of +1.5 and -1.5) were considered significant (see Supplementary Table S2 for the values). Real-time qPCR reactions were carried out in triplicate. Statistical differences were evaluated by nonparametric Mann–Whitney test. p -Values < 0.05 were considered significant.

In fact, *hsp70* was targeted only by PHE and FLT, whereas *COXI* and *COXIII* were not targeted only by FLT. NAP, PHE, and BkF, increased the expression levels of three genes (*hsp60*, *COXI*, and *COXIII*). Moreover, treatment with NAP also down-regulated *hsp26*; the exposure to PHE up-regulated *hsp26* and *hsp70*; FLT is able to up-regulate *hsp26* and *hsp60*, and down-regulate *hsp70*, whereas the exposure to BkF up-regulated *hsp26* and down-regulated *hsp70* (see also Supplementary Table S5).

As shown in Figure 4, among the nine genes analyzed, only one gene (*hsp70*) was not targeted by NAP, PHE, and FLT. In fact, *hsp70* was target only of BkF. Common molecular targets for four contaminants were *HAD-like*, *tcp*, *UCP2*, and *CDC48*, of which only *UCP2* was up-regulated by all treatment, whereas *tcp* and *CDC48* were up-regulated by NAP, PHE, and BkF and down-regulated by FLT; and *HAD-like* was up-regulated by PHE, FLT, and BkF and down-regulated by NAP.

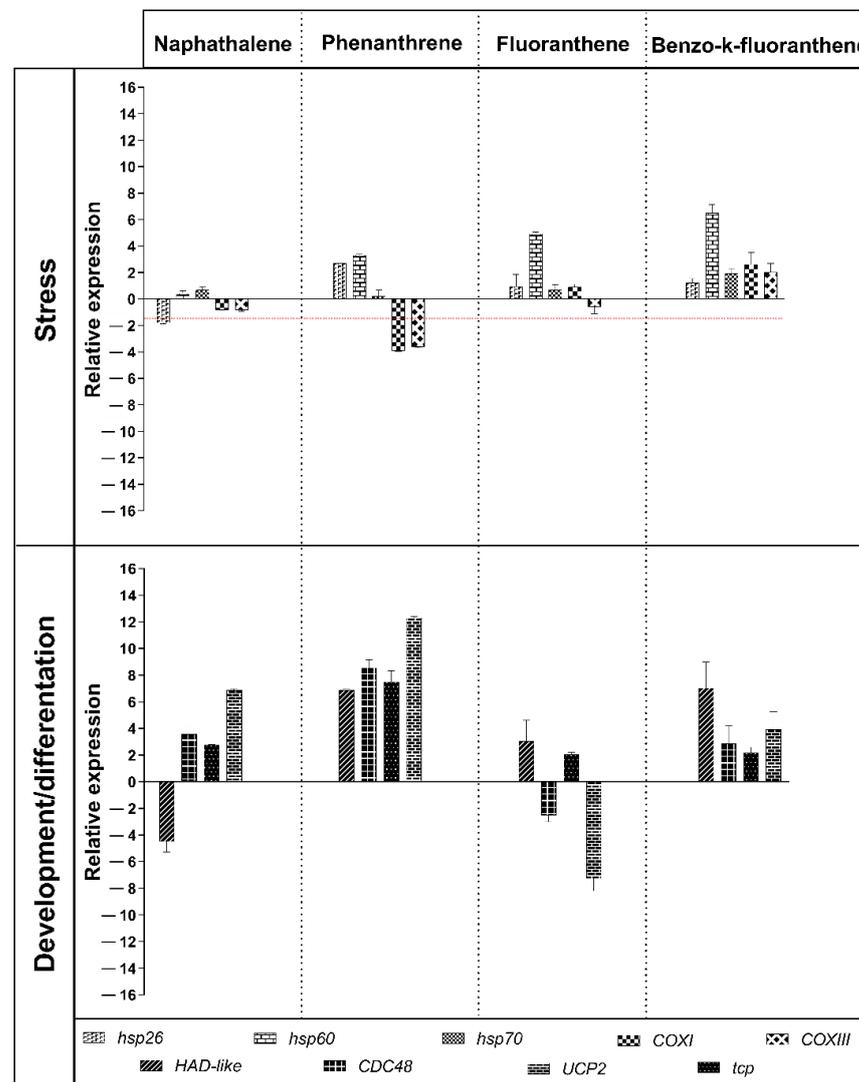


Figure 4. Histograms show the differences in expression levels of five genes involved in stress response. *A. franciscana* nauplii were exposed to naphthalene, phenanthrene, fluoranthene, and benzo(k)fluoranthene at 0.26 mg/L, 1.15 mg/L, 0.81 mg/L, and 84.6 mg/L, respectively. Fold differences greater than ± 1.5 (see red dotted horizontal guidelines at values of +1.5 and -1.5) were considered significant (see Supplementary Table S3 for the values). Real-time qPCR reactions were carried out in triplicate. Statistical differences were evaluated by nonparametric Mann–Whitney test. p -Values < 0.05 were considered significant.

Moreover, *hsp60* was up-regulated by all PAHs with exception of NAP; *hsp26* resulted up-regulated and down-regulated only after PHE and NAP treatment, respectively; and *COXI* and *COXIII* were down-regulated by PHE and up-regulated by BkF (see also Supplementary Table S6 for the values).

4. Discussion

Acute toxicity tests of PAHs showed similar negative impact of single four pollutants (NAP, PHE, FLT, and BkF) on both adults and nauplii. The NAP, PHE, and FLT were able to induce an increase of nauplii death already after 24 h of exposure, whereas the survival of *A. franciscana* was unaffected by exposure to BkF both after 24 h and 48 h. On the basis of lethal concentrations, the FLT (1.30 mg/L) and NAP (1.73 mg/L) appeared to be more toxic than PHE (4.44 mg/L) at 24 h. As shown in Table 2, Bellas et al. [16] showed similar results in both *C. intestinalis* and *P. lividus* embryos. In fact, exposing these two crustaceans to five PAHs for 24 h, they revealed that FLT and NAP were two and six times more toxic than NAP for *C. intestinalis* and *P. lividus*, respectively.

Table 2. Lethal concentration 50% (LC50) values (mg/L) of PAHs.

	PAHs				References
	NAP	PHE	FLT	BkF	
<i>D. magna</i>	7.924 (24 h)	0.458 (24 h); 0.8 (48 h)			[17]
<i>M. galloprovincialis</i>	0.009 (24 h)	0.0002 (24 h)	0.036 (24 h)		[16]
<i>P. lividus</i>	0.012 (24 h)	0.069 (24 h)	0.036 (24 h)		[16]
<i>C. intestinalis</i>	0.001 (24 h)	0.069 (24 h)	0.036 (24 h)		[16]
<i>C. elegans</i>		4.7 (48 h)			[17]
<i>E. fetidas</i>		0.1 (48 h)			[17]
<i>C. tentans</i>	2.81 (48 h)	0.49 (48 h)			[2]
<i>S. capricornutum</i>	2.96 (48 h)	0.94 (48 h)			[2]
<i>N. palea</i>	2.82 (48 h)	0.87 (48 h)			[2]
<i>P. gyrina</i>	5.02 (48 h)				[2]
<i>G. minus</i>	3.93 (48 h)	0.46 (48 h)			[2]
<i>P. promelas</i>	1.99 (48 h)				[2]
<i>S. gairdneri</i>	0.12 (48 h)	0.03 (48 h)			[2]
<i>M. salmonid</i>	0.68 (48 h)	0.25 (48 h)			[2]
<i>A. franciscana</i>	1.73 (24 h); 0.40 (48 h)	4.44 (24 h); 3.07 (48 h)	1.30 (24 h); 0.09 (48 h)		This study

Instead, after 48 h of exposure, the NAP (0.40 mg/L) and FLT (0.09 mg/L) showed a toxicity 7- and 34-times higher than that established for PHE (3.07 mg/L). In comparison with PHE results from this study, the 48 h LC50 was similar to that of *C. elegans* (4.7 mg/L) but lower than those of *D. magna* (0.8 mg/L), *Chironomus tentans* (0.4 mg/L), and *Eisenia fetida* (0.1 mg/L) reported in a previous study (see also Table 2) [17]. When considering adults exposure, after 48 h, the FLT and PHE showed higher toxicity than these of NAP and BkF. Our results suggest that there is a direct relationship between toxicity and aromatic ring number of the tested compounds. Millemann et al. [2] showed the same relationship for the number of aromatic rings and their toxicity. In fact, they revealed that the PAHs with three or four aromatic rings was always more toxic than those with two aromatic rings for each of the nine exposed species (*Selenastrum capricornutum*, *Nitzschia palea*, *Physa gyrina*, *D. magna*, *Chironomus tentans*, *Gaintaurus minus*, *Pimephales promelas*, *Salmo gairdneri*, and *Micropterus salmonid*; see Table 2).

Our study also provides new information on the large-scale genotoxicity information of PAHs on *A. franciscana* adults and embryos. Firstly, when considering the genotoxicity on adults, all five genes were molecular targets of four pollutants, with only the exceptions of *hsp70*, *COXI*, and *COXIII* (Figure 3). When considering the real-time qPCR experiments on nauplii, all nine genes were molecular targets of four pollutants, with the only exception of *hsp26*, *hsp60*, *hsp70*, *COXI*, and *COXIII* (Figure 4). These data suggest that the nauplii

treated with NAP, PHE, FLT, and BkF were very similar, as also shown in the heatmap reported in Figure 5.

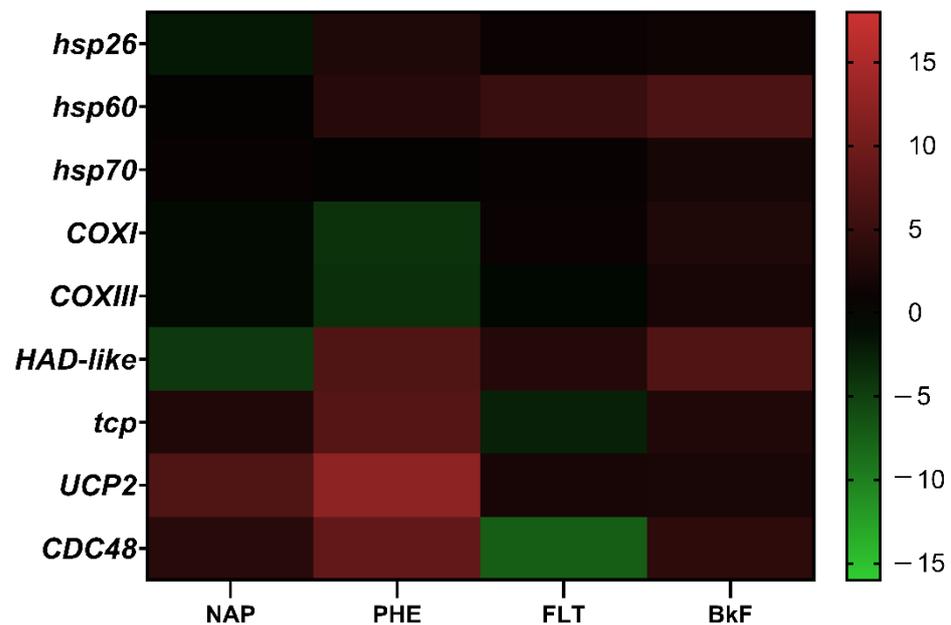


Figure 5. Heatmaps showing the expression profiles and hierarchical clustering of nine genes analyzed through real-time qPCR in nauplii treated with naphthalene (NAP), phenanthrene (PHE), fluoranthene (FLT), and benzo(k)fluoranthene (BkF). Color code: red, up-regulated genes with respect to the control; green, down-regulated genes with respect to the control; black, genes for which there was no variation in expression with respect to the control.

All together, these molecular results revealed that the majority of affected genes in *A. franciscana* were involved in the development processes. In fact, all genes belonging to these classes were affected by the four toxicants. *HAD-like*, *tcp*, *UCP2*, and *CDC48* in *A. franciscana* are involved into molecular mechanisms underlying post-diapause, a common event in diverse taxa from plants to animals [49,52–54]. These data could indicate that PAHs affect some common molecular pathways by changing the normal biological mechanisms, which, in turn, generate death in nauplii and adults. Interestingly, several genes followed by RT-qPCR in the present study were previously found to be functionally interconnected [35,49,52]. In particular, Varó et al. [35] showed that nanoparticles (PS NPs) altered the expression of all genes belonging to the network except for *tcp*, whose relative expression was not significant (Supplementary Figure S1). It is important to underline how the evaluation of the changes in gene expression induced by these toxicants has given the opportunity to uncover some key results that are not easily noticed through observations (i.e., mortality). In fact, the BkF was not able to impact the survival of both nauplii and adults but contemporarily altered the expression level of all tested genes.

It has been widely demonstrated that PAHs are mutagenic, carcinogenic, and teratogenic compounds with long-term effect, especially on human health [55,56]. For these reasons, embryos and larvae of marine invertebrates can be considered as suitable indicators in understanding the toxicological response induced by organic compounds since they are also capable to accumulate high levels of them in their tissues [9,16,19]. Moreover, invertebrate species have a key trophic position in benthic food web, playing the role of intermediate consumers [57]. As a result, the toxicological risk is addressed not only to marine species but also to human beings, which could be exposed to such contamination through the food chain [58,59]. Thus, there is the need to develop early warning systems on consolidated biological models supporting sensitive sub-lethal endpoints. An increase of knowledge on changes of *A. franciscana* genes expression can provide great added values in toxicity assessment. In fact, despite its widespread past use, few studies have been

conducted on the change of gene expression of *A. franciscana* in response to environmental contamination. The identification of molecular pathways in which the targeted genes were involved represents a key step in understanding how crustacean *A. franciscana* protects itself from the stress caused by toxic substances.

In conclusion, genotoxicity may be considered as a possible new biomarker to detect the presence and effects of key environmental pollutants impacting the survival of marine invertebrates. The great simplicity of handling *A. franciscana* in laboratory conditions together with the high sensitiveness of the molecular endpoints could support future applications of this model organism.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w14101594/s1>, Figure S1: Gene network; Table S1: Solubility of PAHs [60–62]; Table S2: Tukey’s test on nauplii survival data after 24 h and 48 h of PAHs exposure; Table S3: Tukey’s test on adults survival data after 24 h and 48 h of PAHs exposure; Table S4: LC50 and 95% confidence intervals calculated after 24 h and 48 h of PAHs exposure; Table S5: Data of gene expression levels in adults; Table S6: Data of gene expression levels in nauplii.

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