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Genotoxicity set up in nauplii and adults of Artemia franciscana 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 and released by anthropogenic activities. They are formed by two or more fused benzene rings. Because of their low water solubility and hydrophobicity, they tend to be adsorbed and accumulated in sediment, where their degradation rate is very low. To the best of our knowledge, no studies have been carried out so far to investigate the in vitro effects of PAHs on *Artemia franciscana*. Artemia is easy to manage at lab scale, but it is not a really sensitive biological model considering the traditional endpoints (i.e., cysts hatching, mortality of nauplii). Thus, we focused on 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 involved in stress response (*hsp26, hsp60, hsp70, COXI* and *COXIII*) and developmental process (*HAD-like, tcp, UCP2* and *CDC48*), revealed molecular effects, with almost all genes targeted by all toxicants.**

Keywords*— *polycyclic aromatic hydrocarbons, short-term effects, toxicity, genotoxicity

I. INTRODUCTION

The crustacean *Artemia franciscana* has been considered as a model species to investigate the ecotoxicological response of marine invertebrates to environmental pollutants [1–3]. 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 [4]. 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) [5]. 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 [1,6]. In fact, in the recent years, the use of this crustacean in ecotoxicology has become increasingly rare [6]. 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 [7–11]. 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

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PAHs and genotoxicity can represent an easy and fast screening method for their ranking [12–14]. 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 x 10^2 mg/L, from 0.41 to 3.9 x 10^2 mg/L, and from 0.025 to 9.4 x 10^1 mg/L for NAP, PHE, FLT, and BkF, respectively) detected in polluted sediments subjected to various pollution sources [12].

II. Materials and Methods

A. Ecotoxicity Test

Acute toxicity test was performed by adding 10 nauplii and 5 adults to each well containing respectively 2 mL and 10 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 (synthetic sea water). The tests were performed in triplicates.The solutions were sampled and analyzed for four PAHs according to Carotenuto et al. [15]. 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. Toxicity data were reported as mean ± 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.

B. RNA extraction, cDNA synthesis and Real Time qPCR

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. Total RNA was extracted and purified using Direct-zol™ RNA Miniprep Plus Kit (ZYMO RESEARCH). 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 (*hsp23, hsp60, hsp70, COXI* and *COXIII;* [16]) involved in stress response were evaluated for adults; for nauplii, the variations in the expression of four other genes (*HAD-like, tcp, UCP2*, and *CDC48*) involved in developmental and differentiation processes were also tested. Undiluted cDNA was used as a template in a reaction containing a final concentration of 0.3 mM for each primer and 1 X SensiFAST™ SYBR Green master mix (total volume of 10 µL) (Meridiana Bioline). PCR amplifications were performed in AriaMx Real-Time PCR instrument (Agilent Technologies, Inc., Santa Clara, CA, USA), according to the manufacturer's instructions. The expression of each gene was analyzed and internally normalized against *GAPDH* [16] using REST software (Relative Expression Software Tool, Weihenstephan, Germany) based on the Pfaffl method [17,18]. Relative expression ratios above 1.5 were considered as significant. Please do not revise any of the current designations.

III. Results

A. Naphthalene, phenanthrene, fuoranthene and benzo-k-fluoranthene toxicity

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 x 10^2 mg/L, 0.41 to 3.9 x 10^2 mg/L, and 0.025 to 94 mg/L, respectively. 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.

After 24 h of nauplii exposure to PAHs, a statistically significant increase in toxicity ($p < 0.0001$) was observed only for NAP, PHE and FLT at the highest tested concentrations (10.1 mg/L for NAP (43% mortality), 223.4 mg/L for PHE (50 % mortality) and 325 mg/L for FLT (70 % mortality)) (Figure 1).

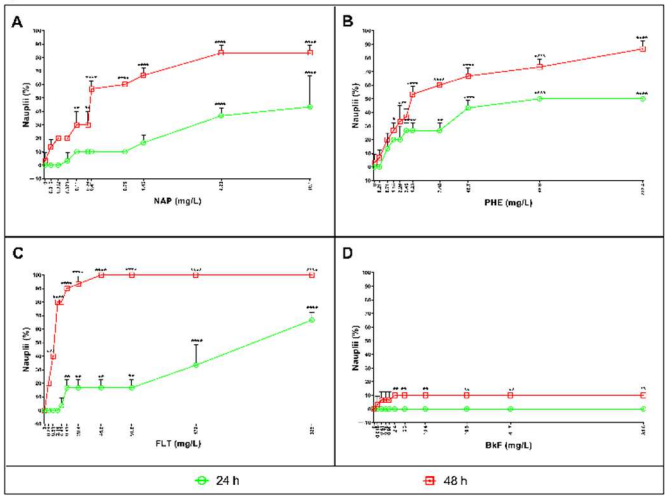

Fig. 1. After 24 h and 48 h, the percentage of dead nauplii in control (0 mg/L) and treated samples with (A) NAP at the concentrations of 0.025, 0.05, 0.1, 0.2, 0.4, 0.5, 1, 2.5, 5, and 10 mg/L; (B) PHE at the concentrations of 0.36, 1, 2, 3, 4, 5, 10, 57.5, 115, and 230 mg/L; (C) FLT at the concentrations 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 ± standard deviation two-way ANOVA by Tukey's test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

After 48 h, mortality became statistically significant respect to control already at lowest concentrations (0.26 mg/L for NAP (30 % mortality; $p < 0.01$), 1.15 mg/L for PHE (26 % mortality; $p < 0.05$) and 0.81 mg/L for FLT (40 % mortality; $p < 0.0001$). 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

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10%) was shown with respect to the control. Moreover, 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.

As reported Figure 2, NAP and PHE and BkF did not affect the survival of *A. franciscana* adults at all tested concentrations after 24 h of exposure.

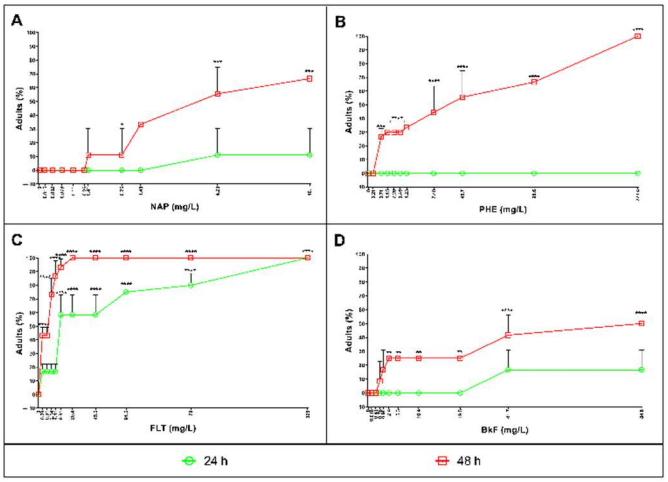

Fig. 2. After 24 h and 48 h, the percentage of dead adults in control (0 mg/L) and treated samples with (A) NAP at the concentrations of 0.025, 0.05, 0.1, 0.2, 0.4, 0.5, 1, 2.5, 5, and 10 mg/L; (B) PHE at the concentrations of 0.36, 1, 2, 3, 4, 5, 10, 57.5, 115, and 230 mg/L; (C) FLT at the concentrations 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 ± 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) and, after 48 h, at 0.29 and 0.81 mg/L, a significant increase of toxicity (about 45%) was displayed with respect to 0 ($p < 0.0001$; Figure 2C). 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); whereas PHE induced an increase of the percentage of dead (about 26.6%) with respect to control already from 0.71 mg/L (Figure 2B). When we considered BkF toxicity (Figure 2D), 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; $p < 0.01$), where a percentage of about 50% was registered.

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.

B. 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).

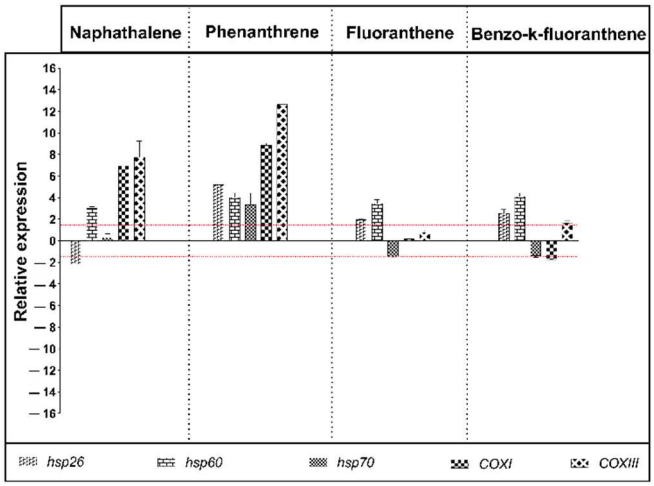

Fig. 3. Histograms show the differences in expression levels of five genes involved in stress response. *A. franciscana* adults were exposed to naphthalene, phenanthrene, fluoranthene, and benzo(k)fluoranthene at 1.45 mg/L, 1.15 mg/L, 0.81 mg/L, and 84.6 mg/L, respectively. Fold differences greater than ±1.5 (see red dotted horizontal guidelines at values of +1.5 and −1.5) were considered significant. 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*.

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.

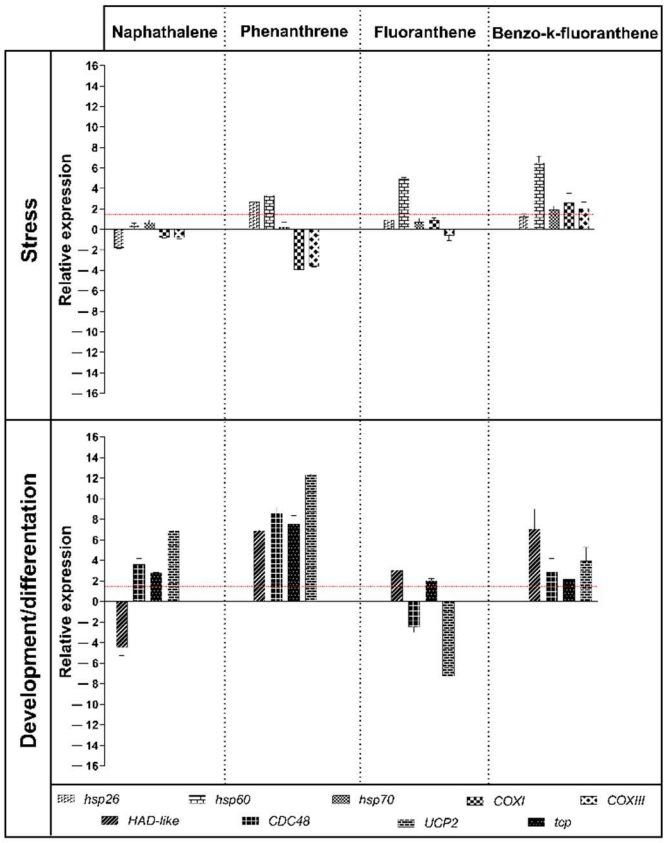

Fig. 4. Histograms show the differences in expression levels of nine genes involved in stress response and development process. *A. franciscana* adults were exposed to naphthalene, phenanthrene, fluoranthene, and benzo(k)fluoranthene at 0.26 mg/L, 1.15 mg/L, 0.81 mg/L, and 84.6 mg/L, respectively. Fold differences greater than ±1.5 (see red dotted horizontal guidelines at values of +1.5 and −1.5) were considered significant. 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.

IV. CONCLUSIONS

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.

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