Ecotoxicol. Environ. Contam., v. 21, n. 1, 2026, 15 - 34
doi: 10.5132/eec.2026.01.03

Original Article

Effects of Crude Oil Water Accommodated Fraction on *Artemia franciscana* and Human Health and Environmental Related Microbes

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Received November 28, 2025; Accept May 13, 2026

Abstract

Marine pollution caused by crude oil spills poses significant risks to ecosystems and human health due to the toxic components of its water-accommodated fraction (WAF), particularly polycyclic aromatic hydrocarbons (PAHs). This study investigated the toxicological effects of WAF derived from the 2019 oil spill in northeastern Brazil on *Artemia franciscana* and medically relevant microorganisms (*Serratia marcescens*, *Staphylococcus epidermidis*, and *Candida tropicalis*). The WAF was prepared from oil-soil mixtures at concentrations of 0.16%, 4%, and 100%. GC-MS analysis of the WAF identified 9 of the 16 EPA priority PAHs in the WAF, with ∑PAHs concentrations of ~20 μg L^{-1} in distilled water. Acute toxicity endpoints (hatching and mortality rates) for *A. franciscana* showed no significant effects, while molecular analyses revealed concentration-dependent alterations in the expression of stress-related genes (e.g., *P26, Dnmt, Hsp26, Hsp40, Hsp70,* and *Hsp90*). Although reactive oxygen species (ROS) levels were assessed using fluorescence microscopy, no detectable changes were observed, suggesting methodological limitations for this endpoint. Microbial assays demonstrated no significant growth alterations for *S. epidermidis* and *C. tropicalis*. However, *S. marcescens* showed increased growth and colony- forming units at the highest WAF concentration, consistent with its polycyclic hydrocarbon- degrading capabilities seen in the literature. These findings suggest that acute exposure to WAF may not elicit immediate toxicological effects in *A. franciscana*, but molecular disruptions could affect long-term resilience. The enhanced proliferation of *S. marcescens* under high WAF concentrations highlights its potential role in bioremediation, albeit with considerations for its opportunistic pathogenicity.

Keywords: Ecotoxicology, gene expression, oil spill, polycyclic aromatic hydrocarbons (PAHs), sublethal effects, Water Accommodated Fraction (WAF).

INTRODUCTION

Marine pollution primarily occurs through anthropogenic activities including industrial discharge, agricultural runoff, shipping operations, and accidental oil spills, which introduce toxic substances into aquatic ecosystems through both point- source and diffuse contamination pathways (Beiras, 2018). These pollutants can cause immediate mortality, sublethal physiological impairments, bioaccumulation in fatty tissues, disruption of endocrine and metabolic processes, and long-term reproductive and developmental dysfunction in exposed organisms (Dat *et al*., 2017; Wang *et al*., 2023). Persistent pollutants,

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such as pesticides and petroleum hydrocarbons, accumulate within these ecosystems, exerting long-term adverse effects on marine life and essential ecosystem functions (Chang *et al*., 2014; Lindeberg *et al.*, 2018; Perrichon et al., 2018). One of the most concerning and majoritarian compounds of crude oil are the polycyclic aromatic hydrocarbons (PAHs) (Mutelet et al., 2002) which are capable of accumulating in the water column despite its hydrophobicity, forming the water accommodated fraction (WAF) of crude oil a complex mix of toxic molecules readily absorbed by exposed organisms and accumulating specially in fatty tissues and the liver, often ending up consumed by humankind, posing a significant health risk (Wang et al., 2023).

Events like the 2019 oil spill along the Brazilian coast released substantial quantities of crude oil in the country's coast, covering over 3000 km and affecting over 900 beaches, introducing widespread contamination with complex, cascading effects on marine ecosystems (Craveiro, 2021; Disner & Torres, 2020a; Lourenço *et al*., 2020, 2023a; Santana *et al*., 2022a; Datta,2023). The 2019 oil spill was not an isolated event in Brazilian coastal history. Previous incidents have highlighted the vulnerability of the Brazilian coastline to petroleum contamination. In Guanabara Bay (Rio de Janeiro), chronic oil pollution from industrial activities and shipping operations has resulted in persistent PAH contamination affecting local fisheries and ecosystems (Silva, 2007; Sotão Neto, 2023). The São Sebastião oil spill (1974) released approximately 11 million liters of crude oil, causing long-term impacts on benthic communities that persisted for decades (Mahiques *et al*., 2022). Similarly, the Ubatuba region (state of São Paulo) has experienced multiple smaller-scale spills that have cumulatively affected marine biodiversity and local fishing communities (Abessa, 2018). These historical precedents underscore the importance of understanding the chronic, sublethal effects of petroleum hydrocarbons beyond immediate acute toxicity, as persistent contamination can exert selective pressure on marine populations and alter ecosystem dynamics over extended timescales WAF components exhibit higher solubility and bioavailability, amplifying their toxicity to both microbial communities and larger marine organisms, disrupts microbial community structures, which affects nutrient cycling and other biochemical processes essential for ecosystem stability (Camacho-Jiménez *et al*., 2023a; Duran & Cravo-Laureau, 2016; Tollette *et al*., 2022; Zhang *et al.*, 2014). Moreover, the PAHs in WAF have a high tendency to bioaccumulate in the tissues of marine crustaceans, causing physiological impairments that affect metabolism, growth, and reproduction, ultimately influencing population dynamics and ecosystem health (Almeida *et al*., 2012a; Almeda *et al*., 2013; Han *et al*., 2014; Cong, 2021; Wang, 2024). The susceptibility of marine crustaceans to PAH-induced impairment is largely attributed to their relatively low metabolic capacity for xenobiotic biotransformation compared to higher vertebrates (Honda & Suzuki, 2020). Consequently, the accumulation of parent PAHs and their reactive metabolites triggers oxidative stress and endocrine disruption, which manifest as sublethal effects on development and recruitment (Wang *et al.*, 2021; Wang *et a*l., 2024). To evaluate these complex toxicological interactions under controlled conditions, *Artemia franciscana* has been extensively utilized as a representative microcrustacean model.

Microcrustaceans—including copepods, cladocerans, and anostracans such as *Artemia* species—are particularly vulnerable to PAH bioaccumulation due to their small body size, high surface-area-to-volume ratio, and basal position in aquatic food webs (Almeda *et al.*, 2013; Han *et al*., 2014). These organisms often lack the metabolic capacity to efficiently biotransform and eliminate lipophilic compounds, leading to the progressive accumulation of PAHs in their tissues (Honda & Suzuki, 2020). Exposure to petroleum hydrocarbons, even at sub-lethal concentrations, results in altered feeding behavior, reduced reproductive output, and increased oxidative stress (Albarano *et al.*, 2022; Marinho da Luz, 2025). As primary consumers, these microcrustaceans serve as critical vectors for transferring contaminants to higher trophic levels, including commercially important fish and, ultimately, humans (Wang *et al.*, 2023). Consequently, *A. franciscana* has become a mainstay in ecotoxicology due to its maintenance efficiency, low cost, and rapid provision of diverse endpoints (Nunes *et al.*, 2016; Schatzer *et al.*, 2024). Recent studies have validated its utility in assessing PAH toxicity; for instance, Albarano *et al.* (2022) established concentration-dependent DNA damage and behavioral alterations in *A. franciscana* exposed to individual PAHs (phenanthrene, naphthalene, fluoranthene, and benzo[k]fluoranthene). Subsequent research by the same group utilized *Artemia* as a sentinel organism to evaluate in situ microcosm remediation of PAHs using nano-zero valent iron and activated carbon (Albarano *et al.*, 2023). Furthermore, the model's versatility for complex mixture assessment was demonstrated by Schatzer *et al.* (2024) through an integrated biochemical, behavioral, and morphological approach. Supporting its specific relevance to petroleum research, Philibert *et al.* (2023)

calibrated an acute toxicity model for *A. franciscana* nauplii to enhance oil spill effect assessments. Collectively, these studies establish *Artemia* spp. as robust models for investigating both the mechanisms and ecological consequences of PAH exposure.

Beyond classic developmental endpoints such as hatching and mortality (Meyer *et al.,* 1982), novelty sensible endpoints are being studied and published using this model (Albarano *et al.*, 2022; Libralato *et al.*, 2016; Schatzer *et al.,* 2024), with genetic expression endpoints being used to understand the response of this organism to toxicants, especially regarding to Heat-Shock Proteins (HSPs). The HSPs response in the context of crude oil PAHs varies with different PAHs or their mixtures; for example, *Hsp70* can be either upregulated or downregulated depending on the PAH combination, presenting a non-obvious response (Albarano *et al*., 2023). Moreover, PAHs present in the crude oil are known to increase generation of ROS (Livingstone, 1998, 2001), leading to oxidative imbalance in the cells, which may cause proteins to fold in animal cells (Kumar *et al*., 2022). The impacts of the PAHs present in the crude oil WAF extend to human health, with one of the ways to cause such alterations being changes to the microbiota, where exposure reduces microbial diversity and alters the abundance of important bacterial groups (Karahan, 2024; Kim *et al.,* 2012).

Conversely, beneficial species like *Bacteroides uniformis*, linked to host urinary metabolites, show reduced abundance when exposed to crude oil PAHs, potentially compromising pathogen defense (Beane *et al.*, 2021). The changes in gut microbiota composition may alter indigenous microbial defense against pathogens, affect host physiology and immune response, and weaken intestinal barrier function, increasing susceptibility to infections and inflammation (Martin *et al*., 2019). The WAF of crude oil serves as an ideal model for studying these effects, as it specifically isolates the bioavailable fraction of petroleum hydrocarbons that bypasses physical oil-droplet interference to directly interact with biological membranes (Philibert *et al.*, 2023; Wang *et al*., 2024). This fraction provides a representative exposure scenario that simultaneously impacts invertebrate sentinel organisms like *A. franciscana* through oxidative stress and developmental toxicity, while concurrently altering the structure of microbial communities with human health relevance (Philibert *et al.*, 2023).

Concurrently, shifts in microbial community dynamics, particularly among opportunistic pathogens, can indicate broader ecosystem disruptions with potential implications for human health (Kim *et al*., 2012; Karahan, 2024). The effects of PAHs and crude oil components on medically relevant microorganisms have been documented in several studies. Kim *et al.* (2012) demonstrated that crude oil, dispersants, and their mixtures alter human fecal microbiota composition in vitro, reducing beneficial bacterial groups and potentially compromising intestinal barrier function. Karahan (2024) reviewed how environmental contaminants including petroleum hydrocarbons affect gut microbiota composition and host health outcomes. Beane et al. (2021) showed that beneficial species like *Bacteroides uniformis* exhibit reduced abundance when exposed to crude oil PAHs, potentially compromising pathogen defense mechanisms.

These findings suggest that petroleum contamination can indirectly affect human health through microbiota alterations, making the assessment of PAH effects on opportunistic pathogens particularly relevant from a public health perspective. *Serratia marcescens*, *Candida tropicalis*, and *Staphylococcus epidermidis* are critical from a medical perspective due to their notable resistance to antibiotics and ability to thrive in healthcare environments. Imbalances in these species can lead to persistent infections, especially in immunocompromised hosts, with increased resistance complicating treatment outcomes and elevating healthcare costs. Consequently, shifts in microbial community dynamics that favor these organisms could pose public health risks by facilitating infections, increasing treatment difficulty, and impacting patient recovery (Cosimato *et al*., 2024; Ferreira *et al*., 2020; Zaric *et al*., 2023).

Accordingly, the selection of these models is predicated on their complementary roles: *A. franciscana* serves as a well-established model for evaluating sublethal and developmental toxicity in aquatic environments, while medically relevant microorganisms function as proxies for assessing how petroleum-derived compounds may influence microbial behavior with implications for human health.

MATERIALS AND METHODS

WAF preparation

The different preparation ratios and solvents for *A. franciscana* and microbial assays were selected based on the specific physiological requirements of each test organism. *A. franciscana* requires 3.5% artificial seawater (ASW) to maintain osmotic balance and normal physiological function, as this species is an obligate halophile adapted to hypersaline environments (Abatzopoulos, 2013). The 10 g/L ratio was selected to

simulate environmentally relevant exposure conditions considering the resurface behavior of crude oil PAHs while ensuring sufficient WAF concentration for toxicological assessment. In contrast, microorganisms were tested in distilled water to avoid osmotic stress interference and because the bacterial and fungal species evaluated are not halophilic, ensuring any differences in growth observed are derived from the presence of PAHs and not from the non-optimum growth medium. The 100 g/L ratio for microorganisms was used due to the innate higher dilutions used in the methodology chosen (where the highest concentration must be diluted to 10% of the source solution), ensuring approximate PAHs concentrations are compatible between assays.

Previous studies have demonstrated that WAF prepared in distilled water yields different PAH concentrations compared to seawater preparations due to salinity effects on hydrocarbon solubility (Schatzer *et al.*, 2025).

Sample origin

Blocks of a sand-oil mixture found at Penedo Beach, located in Alagoas, Brazil (10° 17′ 15″S, 36° 34′ 57″W), were collected on the day they first appeared. The samples were stored under refrigeration at 4ºC in sealed plastic containers, wrapped with paper film and aluminum foil to shield them from light. To accurately assess the oil content without including soil weight, the samples were washed with anhydrous n-hexane (95%, Sigma-Aldrich®), then dried *overnight* in a kiln and weighed before and after washing. This process allowed for determination of the oil portion by removing potential soil contamination. The consistency of organic portion was validated through triplicate extractions, which produced consistent results. Following extraction, the samples showed a 10% mass reduction, attributed to the oil fraction soluble in n-hexane. The mixture of soil and oil was then used to extract the WAF of the crude oil.

WAF extraction and chemical characterization

To prepare the WAF of the crude oil, a modified protocol based on Anderson (1974) ‘s work was followed. Ten grams of the soil-oil mixture was combined with 1 L of 3.5% artificial seawater (ASW – composed of 35 g of sea salt Veromar® – VeroSal Corais per liter of distilled water) for *A. franciscana* assays or 100 g (to account for the initial dilution of 10% required to apply the WAF in the growth medium) with 1 L of distilled water for microorganisms’ assays in Schott bottles and stirred on a magnetic stirrer for 24 hours. The containers were shielded from light with aluminum foil, and the agitation was controlled to keep the vortex at no more than 25% of the total water column height and soil-oil pellets were in constant movement. After 24 hours, the mixture was filtered through a metallic sieve to remove any soil pellets, which were then properly discarded.

The resulting solution, referred to as 100% WAF, was stored in 1 L Schott bottles wrapped in aluminum foil and refrigerated at 4°C for a maximum of 21 days to maintain PAH concentrations (Gallotta et al., 2010) until the moment of the experiments, where it was diluted to 4% and 0.16% to simulate some level of environmental dilution ongoing due to ocean waves activity. The use of different preparation conditions for WAF between *Artemia franciscana* and microbial assays reflects methodological constraints inherent to each biological model. While WAF for *A. franciscana* exposure was prepared using 3.5% artificial seawater to simulate marine environmental conditions, microbial growth assays were conducted in distilled water to avoid confounding effects of salts and ions on culture media composition and microbial proliferation. Similarly, differences in WAF ratios were adopted to ensure organism-specific sensitivity ranges and experimental feasibility. Although these variations limit direct quantitative comparisons between assays, they do not compromise the qualitative interpretation of toxicological and biological responses, as each system was evaluated within its appropriate experimental context.

The analyzed hydrocarbons consisted of the 16 EPA priority PAHs, as well as dibenzothiophene, benzo[e]pyrene, and alkylated homologues (C1–C4-naphthalenes, C1–C3- fluorenes, C1–C3-dibenzothiophenes, C1–C4- phenanthrenes anthracenes, C1–C2-fluoranthenes- pyrenes, C1–C2-chrysenes. The WAF was liquid-liquid extracted at the Instituto Oceanográfico (IO), Department of Physical, Chemical and Geographical Oceanography at the University of São Paulo (USP), with n-hexane (3x30 mL), which extracts the organic (PAHs) fraction present in the WAF. The organic extracts were concentrated to 1 mL through TurboVap II system (Biotage, USA). Internal standards, naphthalene-d8, acenaphthene-d10, phenanthrene-d10, chrysene-d12 e perylene-d12 (PAH), were added prior chromatographic analyses.

The analyses of the PAHs were performed using an Agilent gas chromatograph (model 6890N) coupled to a mass spectrometer detector (MS, model 5973N) equipped with an Agilent J&W 19091S-433 column (HP5-MS: 30 m, 0.25 mm ID, 0.25 µm film thickness). Helium was used as a carrier gas. The

injector temperature was adjusted to 300ºC and splitless mode was adopted. For PAH analyses, the following oven temperature program was used: 40 to 60ºC at a ramp rate of 20ºC min^{-1}; 60 to 270ºC at 5ºC min^{-1}; 270 to 300ºC at 20ºC min^{-1}; and 300ºC for 10 min. Data acquisition was conducted in SIM (selected ion monitoring). Six compounds were identified by matching the retention times with the results from standard compounds, and by the ion mass fragments (m/z). Concentrations of individual compounds were obtained using the internal standard peaks area method and 5-point analytical curves for individual components. The detection limit (DL) was 2.5 ng L^{-1}. Only the WAF obtained in distilled water was characterized because WAF in 3.5% artificial seawater has already been characterized in another study and is available in Supplementary Figure 1 (Schatzer et al., 2025).

***Artemia franciscana* nauplii**

A. franciscana is an anostracan crustacean naturally distributed throughout the Americas, with populations established in hypersaline lakes and coastal salt ponds from the United States to Brazil (Abatzopoulos, 2013). This species has significant economic importance in aquaculture as a live feed organism for larval fish and crustaceans, with global production exceeding 3,500 tons annually (Browdy *et al.*, 2017). Additionally, *A. franciscana* cysts are harvested for the salt production industry as a by-product of solar salt extraction, providing an important source of income for coastal communities. Its ability to produce desiccation-resistant cysts that can be stored for extended periods and hatched on demand makes it particularly valuable for both commercial aquaculture and laboratory research (Nunes *et al.,* 2006, Schatzer 2024). Commercially obtained *A. franciscana* (Artemia do RN®) cysts were kept in a dry and dark environment until the moment of use. To obtain living nauplii, 100 mg of cysts were added to a beaker containing 250 mL of ASW for 24 hours, after which a light source was put in contact with the beaker, attracting phototaxic nauplii which were then removed with a 200 µL micropipette with the very tip of the pipette cut off as to maintain nauplii physical integrity, ensuring the healthy nauplii for subsequent assays.

Hatching and mortality acute assays

To assess the harm posed by crude oil WAF on the hatching and mortality rate of *A. franciscana*, a protocol based on Meyer`s (Meyer *et al.*, 1982) was performed. For hatching rate, 10 commercially obtained (Artemia do RN®) cysts were put into each well of an ELISA plate, in quintuplicates for each concentration and control groups. Once all cysts had been allocated, 200 µL of the testing and control solutions was added to each well. After 48 hours of exposure, the number of living nauplii (defined by being free natant and completely separated from the cyst) was counted. For mortality rate, 5 nauplii with less than 24 hours post-hatching were separated from a beaker in which commercially obtained cysts were put in ASW prior. In quintuplicates, each well of the ELISA plate received 5 living nauplii and their mortality was recorded after 48 hours had elapsed. Nauplii that didn't move for 10 seconds after light mechanical stimuli were considered dead.

Acute exposure of *A. franciscana* for developmental analysis, fluorescence microscopy, and genetic expression studies

Nauplii of *A. franciscana* were cultured in petri dishes containing 10 mL of 3 WAF solutions, 0.16% representing a high dilution setting, 4% as a moderate dilution and stock, or 100% WAF with ASW as control. Circa 150 individuals were placed on each plate to ensure enough individuals were available for each analysis. Plates were left at room temperature following a natural light–dark cycle for 48 hours and covered with an adhesive cover to avoid evaporation of any volatile compounds present in the WAF. After exposure, ~120 nauplii were flash frozen in liquid nitrogen and maintained at - 80ºC to molecular analysis. The remaining nauplii were then treated to fluorescence and scanning electron analysis.

Scanning electron microscopy

Following fluorescence photographic records, nauplii were dehydrated with progressively increasing ethanol concentrations, with 15 minute baths of 15, 30, 50, 70 and 90% concentrations, in which they were kept at 4ºC until the day of SEM analysis. One hour before critical point drying, 90% ethanol was replaced with 100% ethanol. Samples were then critical point dried and gold-sputtered with a 10 nm gold layer. This was made according to the protocol by Schatzer et al. (2024).

***A. franciscana* preparation for fluorescence analysis**

Fluorescence intensity was assessed qualitatively through visual examination by two independent researchers blinded to treatment groups. Images were captured using standardized exposure settings across all samples to ensure comparability. Due to the technical limitations of the epifluorescence microscopy setup, quantitative image analysis using ImageJ or similar software was not feasible, as the

CellROX Green signal exhibited high background fluorescence and uneven illumination across the field of view. Only clearly noticeable changes in green fluorescence intensity relative to control samples were considered positive for ROS induction. Images were evaluated independently by researchers, and consensus was reached through discussion for classification of fluorescence intensity. This qualitative approach represents a methodological limitation, as subtle changes in ROS production may not have been detectable by visual assessment alone. Fluorescence analysis of ROS in nauplii exposed to WAF was done following an adaptation of Schatzer et al.'s protocol (2024). Briefly, nauplii were exposed to CellROX Green Reagent (Thermo Fisher Scientific®) for one hour, after which they were washed with PBS and euthanized with 4% tricaine and fixated on a solution of 4% paraformaldehyde and 3% glutaraldehyde. Nauplii were visualized on a Leica DMI 6000B (Leica Microsystems®) epifluorescence microscope on Cellview plates.

Genetic expression of stress-related genes in *A. franciscana*

Frozen nauplii were thawed in ice and the remainder of any solution was carefully removed. To extract total RNA, 200 µL of TRIzol (Thermo Fisher Scientific®) reagent was added to the sample, followed by mechanical homogenization with a plastic pestle until no tissue fragments were visible and the solution had a slightly opaque appearance. Afterward, 200 µL of chloroform was introduced, and the mixture was vigorously agitated until it appeared milky. Samples were then centrifuged at 12,000 g for 15 minutes at 4°C, producing three distinct layers. The upper aqueous phase, containing RNA, was carefully pipetted into a new tube, where 200 µL of cold isopropanol was added. This solution was mixed again and centrifuged under the same conditions.

After centrifugation, the supernatant was discarded, and the RNA pellet was washed with 1 mL of cold 70% ethanol before undergoing a final centrifugation to remove impurities, resulting in a clear RNA pellet. Once purity was confirmed, the ethanol was removed, and the pellet was resuspended in 50 µL of nuclease-free water, then stored at -80°C. For reverse transcription, RNA concentration was adjusted to 500 ng μL^{-1}, and cDNA synthesis was performed following the manufacturer's instructions with the High-Capacity Reverse Transcriptase Kit (Thermo Fisher Scientific®) in a total reaction volume of 10 µL. All reactions had a final volume of 10 µL; containing 5 µL of GoTaq qPCR Master Mix (Promega®), 0.4 µM of specific primers, 1,5 µL of the synthesized cDNA, and q.s.p. of water Milli-Q Rnase free and had the *At* and *Ef1-α* gene as endogenous control.

The resulting cDNA was diluted to a final volume of 100 µL with nuclease-free water. *Ef1- α* and *At* was chosen as the reference gene. For qPCR, 1 µL each of cDNA and primer mix was added to the reaction, along with GoTaq qPCR Master Mix (Promega®). Reactions were run in technical duplicates on a QuantStudio® 3 Real-Time PCR system (Thermo Fisher Scientific®). Relative gene expression was calculated using the $^{\Delta\Delta}$CT method, with results normalized to the control samples. The sequences and conditions of each gene can be observed in Table 1 and were based on the work of MOHAMMAD *et al.* (2025) except for *Dnmt*, based on Feng's (2007).

Table 1. *A. franciscana* genes studied, their sequence and amplification parameters**.**

Gene	Primer	5'-3' Sequence	Reactions Condition
Hsp26	Forward	CGG AGG ATT TGG TGG TAT GAC	95°C - 15s; 58°C - 30s; 72°C -30s
	Reverse	CCT CAT CAG TTG AGC GTC AC	
Hsp40	Forward	GTG CAT CAG TTG AGC GTC AC	95°C - 15s; 59°C - 30s; 72°C -30s
	Reverse	TGC TGA ACC ATT CCA GGA GC	
Hsp70	Forward	CGA TAA AGG CCG TCT CTC CA	95°C - 15s; 58°C - 30s; 72°C -30s
	Reverse	CAG CTT CAG GTA ACT TGT CCT TG	
Hsp90	Forward	GGT GTG GGT TTC TAT TCT GC	95°C - 15s; 59°C - 30s; 72°C -30s
	Reverse	GCA GCA GAT TCC CAC ACA	
P26	Forward	GCG CGG ATC CAC CAT GGC ACT TAA CCC ATG	95°C - 15s; 57°C - 30s; 72°C -30s
	Reverse	CGC GCC TCG AGT TAA GCT GCA CCT CCT GTC T	
Dnmt	Forward	GAG TGC CGA ACT CAA GAT TGA GA	94°C - 30s; 58°C - 30s; 72°C - 30s; 72°C - 10 min
	Reverse	GCA TTT TGC TGC ACC AAA GAC GA	
At	Forward	GCA GTG GTC TAC AAG GTT TC	95° C - 15s; 60° C - 30s; 72° C -30s
	Reverse	ATC AAA ACG AAG GCT GGC GGT G	
EF1-α	Forward	TCG ACA AGA GAA CCA TTG AAA A	95° C - 15s; 60° C - 30s; 72° C -30s
	Reverse	ACG CTC AGC TTT AAG TTT GTC C	

Microbial liquid growth inhibition and drop plate method

S. marcescens (ATCC 4112) is a Gram-negative, facultatively anaerobic bacillus commonly found in soil, water, and the environment. While not typically part of the normal human microbiota, it is an opportunistic pathogen capable of causing respiratory, urinary tract, and bloodstream infections, particularly in immunocompromised individuals and healthcare settings (Zaric *et al.*, 2023). Notably, *S. marcescens* has demonstrated significant antimicrobial resistance, including carbapenem resistant strains that complicate treatment options (Ferreira *et al*., 2020; Cosimato *et al*., 2024). *S. epidermidis* (ATCC 12228) is a Gram-positive coccus and a prevalent commensal of human skin that plays a protective role by preventing colonization by pathogenic microbes and modulating immune responses (Le *et al*., 2018). However, it is the leading cause of biofilm-associated infections related to indwelling medical devices, with methicillin-resistant strains (MRSE) posing significant treatment challenges (Brescó *et al*., 2017). *C. tropicalis* (IOC 4559) is an ascomycetous yeast and part of the gastrointestinal and mucosal microbiota, contributing to nutrient processing and immune modulation (Di Martino *et al*., 2019). It is increasingly recognized as a significant cause of invasive candidiasis, particularly in immunocompromised patients and those with hematological malignancies, with emerging resistance to azole antifungals complicating therapy (Keighley *et al.*, 2024). These three organisms represent distinct microbial groups (Gram-negative bacteria, Gram-positive bacteria, and fungi) with documented clinical significance and varying susceptibility patterns, making them relevant models for assessing the potential public health implications of PAH-induced microbial community shifts. To assess antimicrobial activity, a liquid growth inhibition assay was performed following the method described by Bulet *et al.* (1993) . A microbial suspension in logarithmic growth phase (10^3 CFU mL^{-1} for bacteria and 10^4 CFU mL^{-1} for fungi) was cultivated in a 96-well ELISA plate containing 10 µL of each treatment and 90 µL of culture medium inoculated with the selected microorganisms. Poor Broth (PB) medium (10 g L^{-1} peptone and 5 g L^{-1} NaCl, pH adjusted to 7.4) was used for bacteria, while Potato Dextrose Broth (PDB) (6 g L^{-1} potato infusion and dextrose) was used for *C. tropicalis*. Microbial strains (ATCC 12228 for *S. epidermidis*, ATCC 4112 for *S. marcenscens*, and IOC 4559 for *C. tropicalis*) were maintained in stocks at -80°C before tests. Each plate contained negative control wells containing only growth medium and ultra-pure water to ensure no contamination of growth medium. Positive controls were composed of 10 µL ultra-pure water and 90 µL of cultured suspension. Plates were then incubated under agitation in a bacteriological incubator for 24 hours. After this time had elapsed, absorbance of each well with a 595 nm wavelength was measured in a microplate reader (Thermo Scientific Multiskan, Go, Thermo Fisher Scientific®) and the average of the positive control was considered 100% growth, with changes being represented as percentage of the control.To better assess changes to the growth of *S. marcescens*, a drop plate test followed the liquid growth medium test. Briefly, 10 µL from the wells of the ELISA plate containing S. *marcescens* for the highest concentration of WAF and a control well was pipetted out after the reading and diluted in 990 µL of PB medium, and from the resulting solution, 100 µL was removed and diluted in 900 µL of PB, and so subsequently for 3 more times, resulting in a dilution of 105 of the solution of the ELISA well. The solution was then used to perform a drop plate test. In a petri dish containing LB agar, 10 µL of the 100% WAF dilution from the previous test prepared was added to the top first quarter of the petri dish side by side, with 9 replicates for each group (100% WAF vs. control) on each half of 3 plates. The plates were then inclined to a 45º angle in relation to the flat surface, allowing the drops to run across the plate, spreading the bacterial seeded solution in a straight line. Upon approaching the final quarter of the plate, it was returned to a flat position to avoid sample mix. The plates were then incubated in a bacteriological incubator at 37ºC for 24 hours and the number of CFUs was counted on the next day.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA), with data presented as mean ± standard error of the mean (SEM) across experimental groups. A one-way ANOVA was performed for each experiment, followed by Dunnett's post-hoc test to compare each treatment group with the control, using a significance level of 0.05.

RESULTS AND DISCUSSION

WAF characterization in distilled water

The concentration of PAHs in the WAF in distilled water are summarized in Figure 1. Among them, alkyl-phenanthrene-anthracenes and fluorene were identified as the predominant compounds, constituting 32% and 27% respectively of the total PAH content in the analyzed sample, with Σ EPA

Priority-PAHs = 0.52 µg L^{-1} and Σ PAHs = 20.62 µg L^{-1}. As demonstrated in Figure 1, the profile of PAHs in the WAF is consistent with the profile found in other oil spill cases, such as the Deepwater Horizon (DWH) platform (Barron *et al*., 2003, 2020; Clement & John, 2022; Faksness *et al*., 2015; Perez-Umphrey *et al.*, 2018).

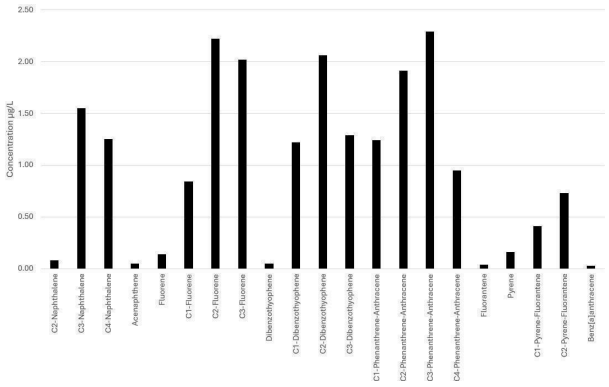

Figure 1. PAHs concentration in one sample of the WAF in distilled water. Data obtained through gas chromatography coupled with mass spectrometer.

Despite the markedly lower concentrations of PAHs detected in the WAF prepared with distilled water—approximately ten times less than those observed using 3.5% artificial seawater (see Supplementary Figure S1), as reported by Schatzer *et al*. (2025)—the chromatographic profile remains similar, particularly in the predominance of alkylated derivatives. In the sample analyzed in the current work, phenanthrene-anthracene substitutes comprised circa 31% of the total PAHs found, followed by fluorene and its substitutes with 25%. Substituted compounds, although often underestimated in traditional risk assessments (Morshead *et al.*, 2025), have shown higher environmental persistence (Cummings *et al.*, 2021) and, in many cases, enhanced toxicity when compared to their parent analogues (Fallahtafti *et al*., 2012; Cummings *et al*., 2021) and are more readily bioaccumulated, inducing significant oxidative stress, genotoxicity, and developmental abnormalities in aquatic invertebrates (Honda & Suzuki, 2020; Marinho da Luz, 2025).

The presence of 9 out of the 16 EPA-priority PAHs, combined with this complex mixture of alkylated hydrocarbons, suggests that even low-level exposure may pose chronic ecological and toxicological risks, especially in coastal areas repeatedly exposed to oil residues, where remobilization and slow degradation processes sustain prolonged contamination (EPA, 2007; Wang *et al*., 2024). The persistence of such compounds in shoreline waters increases the likelihood of human exposure via dermal contact, inhalation of volatile hydrocarbons, and consumption of contaminated seafood—pathways that have been associated with health effects ranging from dysbiosis and allergic reactions to systemic toxicity (Cousin & Cachot, 2014; Engraff, 2011; Magalhães *et al.*, 2022; Mohammed *et al.*, 2022; Vagi *et al*., 2021; Wang *et al.*, 2023, 2024). Public health studies following major oil spills have documented increased incidence of dermatological conditions, gastrointestinal symptoms, and psychological distress among exposed coastal populations (Cousin & Cachot, 2014; Engraff, 2011; Vagi *et al.*, 2021). This reinforces the need to investigate not only acute toxicity endpoints, but also sublethal effects using concentrations that might not be considered environmentally relevant (Berríos-Rolón *et al.*, 2025; Eisler, 1987) across longer time frames. The ubiquitous presence of PAH contamination throughout the globe, even at low concentrations, may subtly alter environments over time, imposing selective pressure on sensitive organisms (Parmar *et al*., 2024; Teixeira *et al.*, 2024) and leading to long-term alterations in species richness that might otherwise go unexplained or unforeseen (Berríos-Rolón *et al*., 2025).

Hatching and mortality assays

Though the Crustacea group are knowingly sensitive to the effects of crude oil's polycylic aromatic hydrocarbons (Honda & Suzuki, 2020), the effects on WAF of the crude oil spilled on the Brazilian coast on *A. franciscana* hatching and mortality didn't incur in significant changes from the control group. The graphs can be observed in Figure 2.

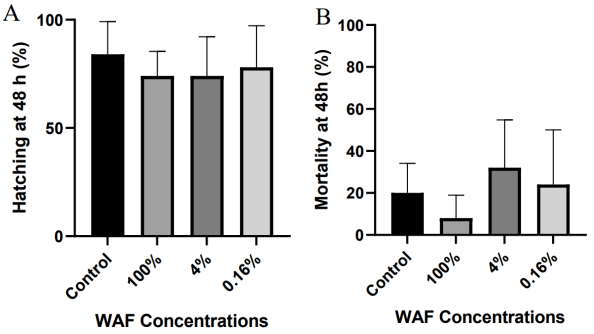

Figure 2. Hatching and mortality changes to *A. franciscana* exposed to concentrations of 0.16, 4 and 100% WAF of crude oil. A: Hatching rate [F (3, 16) = 0.4214, p = 0.7402]. B: Mortality rate [F (3, 16) = 1.316, p = 0.3038]

The absence of significant changes in the hatching and mortality rates of *Artemia franciscana* exposed to crude oil WAF indicates a tolerance of this species at the tested concentrations (Nunes *et al.*, 2006; Libralato, 2016), contrasting with several reports of the acute toxicity of crude oil components, particularly petrogenic PAHs, which target developmental processes in aquatic organisms (Incardona *et al*., 2011; Hodson, 2017). Studies using the ghost shrimp *Palaemon serenus* demonstrated much higher acute toxicity for

dispersed crude oil than WAF, with 96-h LC_{50} values of 258,000 ppm for WAF but significantly lower values for dispersed oil due to increased bioavailability of toxic components (Gulec & Holdway, 2000; Khabib *et al*., 2022). Similarly, work on key Arctic species such as *Calanus glacialis* found LC_{50} values ranging from 1.6 mg L^{-1} to 4.0 mg L^{-1} for WAF preparations, depending on hydrocarbon composition and environmental conditions (Gardiner *et al.*, 2013a,b).

Notably, the findings align with previous observations in larvae of Australian bass (*Macquaria novemaculeata*), where WAF from crude oil exhibited relatively low toxicity compared to dispersed oil. Dispersants enhance the solubility and dispersion of hydrocarbons, facilitating absorption and ingestion of crude oil droplets and particles by exposed organisms (Almeda *et al*., 2014). However, without dispersants in the present study, the exposure conditions likely limited the bioavailability of toxic fractions such as PAHs, minimizing acute effects. This pattern is consistent with observations in *Litopenaeus vannamei*, where WAF preparations containing benzo[a]pyrene were classified as practically non-toxic under EPA criteria, despite measurable PAH concentrations (Asadi & Khoiruddin, 2017). Moreover, broader ecotoxicological reviews emphasize that PAH toxicity is highly dependent on environmental factors and organismal traits, including lipid content, developmental stage, and metabolic capacity (Berríos-Rolón *et al*., 2025; Dupuis & Ucan-Marín, 2015). The effects of PAH mixtures on crustacean development have been documented across various species and exposure scenarios. In copepods (*Tigriopus japonicus*), exposure to binary and ternary PAH mixtures resulted in synergistic toxicity, with combination effects exceeding those predicted by individual compound toxicity (Han *et al*., 2014).

Similarly, studies on *Daphnia magna* demonstrated that PAH mixtures in the presence of suspended sediment enhanced toxic effects due to increased bioavailability (Zhang *et al*., 2014). Barron *et al*. (2020) reviewed long-term ecological impacts from major oil spills, documenting sublethal effects on crustacean populations including reduced reproductive success and altered population structure even in the absence of acute mortality. These findings align with the current observation that while acute endpoints (hatching and mortality) were not significantly affected by WAF exposure, the sublethal molecular disruptions observed (Section 3.5) suggest the possibility of longer-term physiological consequences. However, as the exposure period in this study was limited to 48 hours, extrapolation to population-level effects remains speculative. Further investigations with extended exposure durations and multigenerational designs would be necessary to determine whether these molecular alterations translate into ecologically relevant outcomes such as reduced fitness, reproductive success, or population resilience.

Development analysis through SEM

Crude oil and crude oil components exposure can significantly affect the anatomical development of crustaceans, often leading to sub-lethal and developmental abnormalities. To investigate if crude oil WAF in the concentrations tested induce any malformations or abnormalities in development, nauplii were exposed and images obtained through SEM can be seen in Figure 3.

In other works, exposure to crude oil has caused abnormalities during embryonic and larval stages, such as in the experiments on the gelatinous zooplankton, in which structural development of the larval forms was affected (Abatzopoulos, 2013; Almeda *et al*., 2013). Although these types of studies, as in gelatinous zooplankton, have shown that crude oil exposure could drastically disturb the developmental process in marine larvae, our observations did not present morphological changes in the *A. franciscana* exposed to WAF of crude oil. This absence of morphological abnormalities agrees with studies which reported developmental resilience for some crustacean species against sub-lethal exposures (Philibert *et al.*, 2023). In the case of petroleum hydrocarbon exposure to American lobster larvae, Capuzzo *et al.* (1984), for example, noticed that even though lipid metabolism and energetic reserves were changed, physical deformities were not present in the crustaceans used.

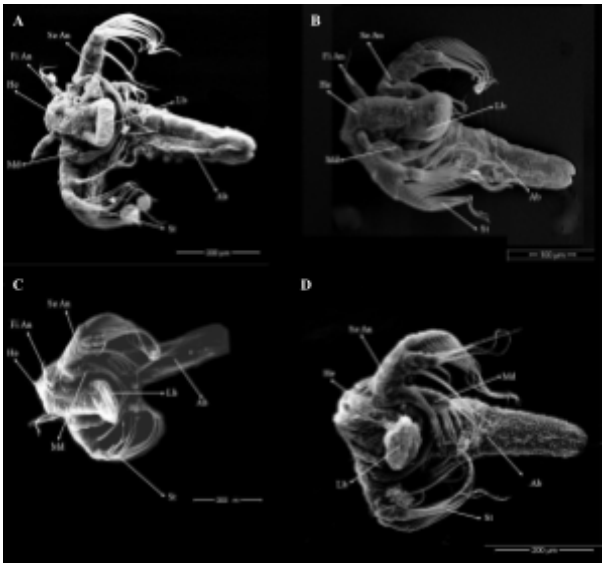

Figure 3. Scanning electron microscopy of nauplii exposed for 48 hours to different WAF concentrations. A: Control. B: 0.16%. C: 4%. D: 100%. Ab: Abdomen. Fi An: First pair of Antennae. Se An: Second pair of Antennae. He: Head. Lb: Labrum. Md: Mandible. St: Setae.

Similarly, a review by Barrento *et al.* (2021) on brachyuran crabs highlighted that larval stages are generally the most sensitive to petroleum hydrocarbons, with sub-lethal effects such as growth impairment, delayed molting, and metabolic alterations, even in the absence of visible deformities. In *Macrobrachium borellii*, a fresh water shrimp, Pasquevich *et al.* (2013) reported that sub-lethal exposure to the water-soluble fraction of crude oil significantly altered the expression of proteins involved in energy metabolism and detoxification such as GAPDH and crustacyanin-like CTC without inducing morphological abnormalities. Under controlled conditions, other variables can also affect the results: different exposure times and different WAF preparation methods, which poses a challenge to reproducibility of results around different research groups (Wheeler *et al*., 2020). Moreover, the lipophilic capabilities of PAHs results in the accumulation of these substances in fatty tissues, allowing them to persist in the organism and induce chronic physiological stress, such as metabolic disruption, without necessarily producing visible morphological abnormalities (Honda & Suzuki, 2020).

ROS assessment through fluorescence microscopy

ROS generation is a major mechanism of toxicity in crustaceans and other marine species exposed to PAHs from crude oil (Livingstone, 1998, 2001). To assess ROS formation in *A. franciscana* nauplii exposed to WAF in different concentrations, the fluorescence assay based on Schatzer's (Schatzer *et al.*, 2024) was performed, with the results seen in Figure 4.

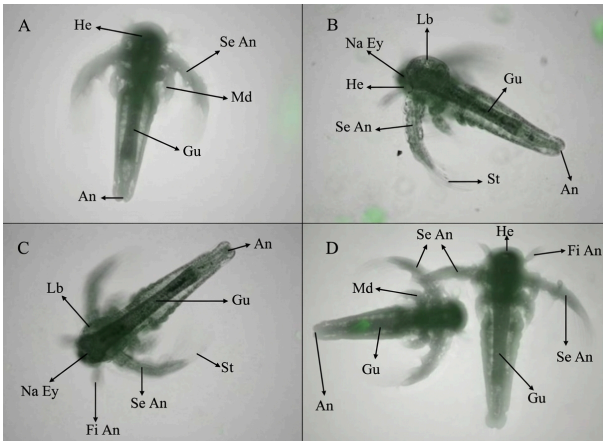

Figure 4. Nauplii exposed for 48 hours to crude oil WAF dyed with CellRox observed under fluorescence microscopy. A: Control. B: 0.16%. C: 4%. D: 100%. Bright green spots are due to equipment artifact and are not considered as changes to ROS production. Fi An: First pair of Antennae. Se An: Second pair of Antennae. He: Head. Lb: Labrum. Md: Mandible. St: Setae. An: Anus. Na Ey: Naupliar Eye. Gu: Gut.

Exposure to crude oil significantly alters the oxidative balance in crustaceans. Various studies have presented specific responses depending on the form and concentration of the crude oil used. For example, copepods and other crustaceans exposed to WAFs of crude oil most often exhibit disturbances of the oxidative balance as a consequence of ROS overproduction (Fu *et al*., 2012; Yin *et al.*, 2007; Yuewen & Adzigbli, 2018). In the present work, fluorescence intensity has not increased or decreased as expected, though previous experiments had pointed towards an activation of antioxidant defense (data not shown). Despite the known activation of antioxidant defense enzymes in response to crude oil WAF exposure (Livingstone, 1998, 2001; Shimada, 2006), no significant differences in fluorescence were observed between the treatment groups using the CellROX fluorescence assay. This suggests that fluorescence microscopy, as employed in this study, may not be a suitable technique for reliably detecting ROS induction under these conditions. While CellROX is commonly used to measure ROS production in various organisms, its sensitivity can vary depending on the concentration and duration of ROS exposure, as well as the specific characteristics of the biological system being studied. In the case of *A. franciscana* nauplii, it is possible that the ROS levels generated by crude oil WAF exposure were not high enough to produce a detectable fluorescence signal, or that the antioxidant response was sufficiently effective to prevent ROS accumulation to levels detectable by this assay. Thus, alternative techniques, such as direct ROS quantification or more sensitive probes, may be more appropriate for assessing oxidative stress in these organisms under such circumstances.

Expression of stress-related genes in *A. franciscana* exposed to WAF

In invertebrates, genetic expression is used as a tool by ecotoxicologists as biomarkers to assess if a given pollutant is affecting a certain environment. In *Artemia* spp., the expression of *Hsps* have been studied in response to PAHs in simple, binary or ternary mixtures (Albarano *et al.*, 2022, 2023), with results ranging from synergistic to antagonistic effects, reflecting the high adaptability of this gene family to environmental pollutants. The expression of *P26*, *Dnmt*, *Hsp26*, *Hsp40, Hsp70* and *Hsp90* of *A. franciscana* exposed to different concentrations of WAF are presented in Figure 5.

Paramount to the diapause characteristic of this organism, the variations in *P26* expression, influenced by environmental or chemical factors, significantly impact the organism's stress tolerance and developmental stability (King *et al*., 2013; Malitan *et al*., 2019; Onyena *et al*., 2024a).

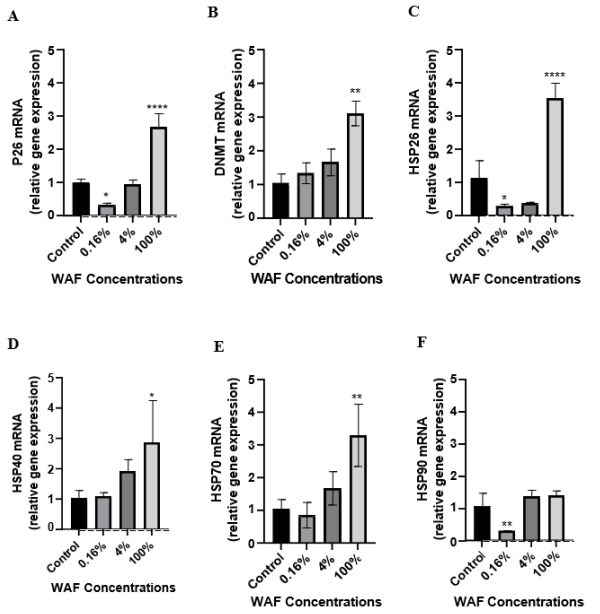

Figure 5. Resistance genes relative expression compared to the constitutive genes EF1-α and At. A: *P26* mRNA expression. $F_{[3, 8]} = 60.00$, $P < 0.0001$; B: *Dnmt* mRNA relative gene expression. $F_{[3, 8]} = 21.98$, $P = 0.0003$; C: *Hsp26* mRNA relative gene expression. $F_{[3, 8]} = 55.96$, $P < 0.0001$; D: *Hsp40* mRNA relative gene expression. $F_{[3, 8]} = 3.848$, $P = 0.0566$; E: *Hsp70* mRNA relative gene expression. $F_{[3, 8]} = 10.50$, $P = 0.0038$; F: *Hsp90* mRNA relative gene expression. $F_{[3, 8]} = 14.48$, $P = 0.0013$. ****: p<0.0001; **: p<0.01; *: p<0.05. ANOVA followed by Dunnett's.

The diminished expression in *A. franciscana* exposed to the lower concentration could result in earlier diapause termination which in turn might affect the general survival of the organism. Though hatching assays resulted in no difference between the concentration that lowered expression of *P26,* the real viability of these nauplii (i.e. the correct development of adult structures and systems as well as reproduction capabilities) is still unknown, with chronic testing to low WAF concentrations being needed to enlighten the consequences of this inhibition. The increase in the higher concentration of WAF tested appears as a compensatory mechanism in order to further protect *A. franciscana* against the harm posed by the toxic components present in WAF (Tan & Macrae, 2018).

The implications of altered *P26* expression extend beyond individual-level effects to potential population-level consequences. This could result in reduced survival of emerging nauplii, decreased reproductive success, and ultimately diminished population resilience over multiple generations. The dose-dependent reversal in *P26* expression, from inhibition at 0.16% WAF to upregulation at 100% WAF, reveals a complex biphasic stress response that warrants careful consideration. At low WAF concentrations, the organism may fail to detect or mount a defense response against chemical stress, a phenomenon known as "silent toxicity" or sub-threshold exposure (Berríos-Rolón *et al.*, 2025).

In this scenario, PAHs and other hydrocarbons may interact with cellular components without triggering protective mechanisms, allowing cumulative damage to proteins and cellular structures to occur unchecked. This lack of response should not be interpreted as absence of harm; rather, it may represent the most insidious form of toxicity, where cellular defenses remain dormant while damage accumulates. In contrast, at high WAF concentrations (100%), the stress signal likely exceeds a critical threshold that activates cellular defense mechanisms, including upregulation of *P26* as a compensatory response. This threshold-dependent activation is consistent with the hormetic dose-response model observed in toxicology, where low doses may inhibit biological processes while high doses stimulate protective responses (Calabrese, 2005). The upregulation of *P26* at high concentrations may represent an attempt to stabilize proteins and prevent aggregation under conditions of severe chemical stress, even though this response may come at an energetic cost to the organism. This biphasic pattern has important implications for environmental risk assessment. Traditional toxicity testing often focuses on high-dose effects, potentially missing the subtle but ecologically significant impacts of chronic low-dose exposure. The observation that the lowest WAF concentration tested (0.16%) inhibited *P26* expression while higher concentrations stimulated it suggests that environmentally relevant exposure levels such as those resulting from continuous low-level seepage or residual contamination may pose greater long-term risks than predicted by acute toxicity data alone. Although acute toxicity endpoints (hatching and mortality) showed no significant differences between treatment and control groups (Section 3.2), the molecular disruption of a gene critical for cryptobiotic survival suggests that sublethal WAF exposure may impose hidden costs that manifest only over longer timescales.

The *Dnmt* gene encodes a DNA methyltransferase that exhibits dual activity: weak DNA methyltransferase function and robust RNA methyltransferase activity, particularly targeting cytosine residues in aspartic acid transfer RNA playing a pivotal role during diapause, a state of developmental arrest characterized by extreme stress tolerance, as its expression significantly increases during the resumption of cyst development (Feng, 2007). This regulation likely supports genomic stability and stress resilience by methylating RNA substrates and participating in gene expression modulation (Feng *et al.*, 2007; Wikumpriya *et al.*, 2023). The observed threefold increase in *Dnmt* expression in *A. franciscana*

exposed to higher concentrations of WAF may reflect an adaptive mechanism to mitigate cellular damage induced by environmental stressors. Increased methylation activity could stabilize RNA structures and enhance the efficiency of translation under toxic conditions, thereby ensuring critical protein synthesis. However, prolonged overexpression might lead to unintended epigenetic changes, potentially disrupting gene regulation and developmental processes (Liu *et al*., 2022; Navarro‐Martín *et al*., 2023).

The expression of *Hsp40* and *Hsp70*, crucial molecular chaperones that work in tandem in stress responses, significantly increased in *A. franciscana* exposed to the highest concentration of crude oil WAF. *Hsp70* is central to refolding damaged proteins and preventing aggregation under stress, while *Hsp40* acts as a cochaperone, enhancing the ATPase activity of *Hsp70* and directing substrates to it (Foster *et al.*, 2015; Li *et al.,* 2016). In the presence of WAF, *Hsp40* exhibited a twofold increase and *Hsp70* was similarly upregulated, despite the absence of thermal fluctuations which is a classic inducer of *Hsp40* and *Hsp70* (Foster *et al*., 2015; Li *et al.*, 2016). This suggests that chemical stress alone can elicit a robust molecular defense response, in accordance with studies in other aquatic organisms confirm that *Hsp70* and *Hsp40* respond to other environmental stressors (Lencioni *et al*., 2016; Rhee *et al.*, 2009; Rowarth & MacRae, 2018). *Hsp90*, as other Hsps from its family, has the maintenance and upkeep of the cell as its main function through a series of processes: protein folding, stabilization of client proteins under stress conditions, and regulation of signaling pathways critical for cellular homeostasis and survival. Its role as a molecular chaperone becomes particularly evident during environmental stress, where it protects cells from damage by managing protein denaturation and aggregation (Gusev *et al*., 2006; Makhnevych & Houry, 2012; Zhang *et al*., 2009). The significant decrease in the expression of this gene in the lowest concentration (0.16%) tested points towards severe impacts on the life processes of this microcrustacean, possibly affecting its development in the long term and its capacity to manage stressful environments, which is a key ecological characteristic of this species. Due to the stable expression in higher concentrations and similar significant decrease of *P26* and *Hsp26*, the harmful substances present in WAF in low concentrations might be enough to affect the expression of these proteins, which is corrected once concentrations are high enough to trigger cellular defenses against toxicants, which in turn seem to increase in a compensatory to all Hsps except *Hsp90*. The low concentrations should be further assessed to identify harm posed to organisms in a chronic manner, given that ocean seepages occur persistently and affects the marine environment with low concentrations of crude oil and crude oil derivatives continuously (Kvenvolden & Cooper, 2003; Dong *et al.*, 2022).

Microbial growth assessment

Crude oil and its derivatives can affect the human microbiome, altering population density, favoring some groups of microorganisms while posing challenges to others (Kim *et al.*, 2012), thereby impacting health through indirect routes. The changes in growth in liquid medium of medically relevant microorganisms *S. epidermidis*, *S. marcescens*, and *C. tropicalis* can be observed in Figure 6.

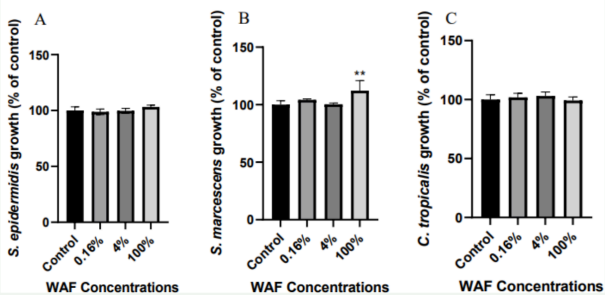

Figure 6. Growth in liquid medium for different microorganisms. A: *S. epidermidis* growth in liquid medium. F[3, 16] = 2.943, P = 0.065. B: *Serratia marcescens* growth in liquid medium. F[3, 16] = 7.292, P = 0.0027. C: *Candida tropicalis* growth in liquid medium. F[3, 16] = 1.252, P = 0.324. ** p < 0.005.

S. epidermidis, *S. marcescens*, and *C. tropicalis* are microorganisms commonly associated with human microbiota and environmental niches. *S. epidermidis* is a prevalent skin commensal that plays a protective role by preventing colonization by pathogenic microbes and modulating immune responses (Le *et al.*, 2018). However, overgrowth or introduction into sterile areas, such as during medical device implantation, can lead to biofilm-associated infections (Brescó *et al*., 2017; Le *et al.*, 2018). *S. marcescens* is typically found in the respiratory and urinary tracts, as well as in soil and water, and while not usually part of the normal microbiota, its presence in small numbers is not harmful (Zaric *et al*., 2023). Its overgrowth can result in opportunistic infections, particularly in immunocompromised individuals, leading to conditions such as pneumonia or, in rare cases, endocarditis (Wilkowske, 1970; Kim *et al*., 2013; Kamali *et al.*, 2024). *C. tropicalis* is part of the gastrointestinal and mucosal microbiota, contributing to nutrient processing and immune modulation (Di Martino *et al.*, 2019; Doan *et al.*, 2024; Keighley *et al.*, 2024). When its population diminishes, competitive inhibition against pathogenic organisms may be reduced, whereas overgrowth can lead to invasive candidiasis, particularly in

immunocompromised individuals (De Barros *et al*., 2018; Keighley *et al.*, 2024).

The effects of PAH mixtures on medically relevant microorganisms have been investigated in several contexts. Kim *et al*. (2012) demonstrated that crude oil and dispersant mixtures alter the growth dynamics and community composition of human fecal microbiota in vitro, with selective suppression of beneficial Bacteroides and Firmicutes. Beane *et al.* (2021) showed that PAH exposure reduces the abundance of *Bacteroides uniformis*, a species associated with host metabolic regulation and pathogen defense. Karahan (2024) reviewed how environmental PAH contamination affects gut microbiota composition, potentially compromising intestinal barrier function and increasing susceptibility to opportunistic infections. Together, these findings indicate that petroleum hydrocarbon exposure can exert selective pressure favoring hydrocarbon-degrading organisms such as *S. marcescens*, while suppressing beneficial commensal species, with implications for both ecosystem function and human health.

In this context, some of the microorganisms evaluated in this study have also been reported as active agents in the bioremediation of crude oil-derived PAHs. Hydrocarbonoclastic strains of *S. marcescens* possess metabolic pathways enabling the degradation of aromatic compounds and the use of petroleum hydrocarbons as sole carbon sources, with high removal efficiencies reported in microbial consortia (Saibu *et al.*, 2022). Similarly, *C. tropicalis* has demonstrated substantial biodegradation capacity in marine environments, including high removal rates of naphthalene and other petroleum-derived compounds (Hegazy *et al.,* 2024). Although less frequently reported, environmental isolates of *S. epidermidis* have also shown the ability to degrade hydrocarbons such as diesel and burned engine oil, suggesting a potential auxiliary role in bioaugmentation strategies.

Our results indicate an increase in *S. marcescens* CFUs at 100% WAF concentration, consistent with its capacity to utilize petroleum hydrocarbons as substrates (Peekate & Ogolo, 2024), as reflected by the higher number of colonies observed in the drop plate assay (Figure 7).

The significantly higher CFU counts of *S. marcescens* observed at 100% WAF compared to the control demonstrate its ability to grow under highly contaminated conditions by metabolizing hydrocarbons. This is supported by previous studies showing that *S. marcescens* can degrade a wide range of petroleum products and produce biosurfactants that enhance hydrocarbon bioavailability (Zhang *et al*., 2021), as well as significantly reduce total hydrocarbon concentrations in contaminated soils (Peekate & Ogolo, 2024).

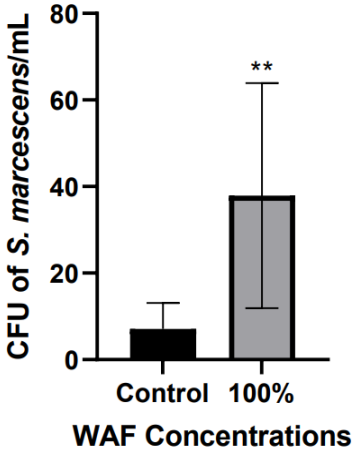

Figure 7. *S. marcescens* colony-forming units in the drop plate method. F[8, 8] = 18.75, P = 0.0004, ** p < 0.0005.

These characteristics highlight its potential applicability in bioremediation strategies for petroleum-contaminated environments. However, the proliferation of *S. marcescens* also raises important public health considerations, as it is an opportunistic pathogen. Therefore, while its metabolic versatility supports its use in hydrocarbon degradation, its application in bioremediation must be carefully managed to avoid unintended risks, particularly in environments with potential human exposure (Wilkowske, 1970; Zaric *et al*., 2023).

The findings of this study raise important questions about the long-term consequences of chronic WAF exposure for both *A. franciscana* populations and microbial community dynamics. For *A. franciscana*, the concentration-dependent dysregulation of stress-response genes (*P26*, *Hsp90*) at low WAF concentrations suggests that chronic exposure to environmentally relevant PAH levels may progressively erode population resilience. Reduced *P26* expression could compromise diapause maintenance, leading to premature cyst hatching during unfavorable conditions and reduced survival of subsequent generations (King *et al*., 2013). Similarly, decreased *Hsp90* expression may impair the organism's ability to manage protein folding under additional stressors, potentially reducing fitness in dynamic coastal environments, while also possibly making the organism less fit to thrive in its classical environment of natural salines. These sublethal effects, while not immediately lethal, could manifest as reduced population growth rates, altered sex ratios, or increased susceptibility to predation and disease over extended timescales.

For microbial communities, the selective proliferation of *S. marcescens* under high WAF concentrations suggests that chronic petroleum

contamination may drive community shifts favoring hydrocarbon-degrading opportunists. While this presents potential benefits and candidates for bioremediation, the displacement of beneficial commensal species and proliferation of resistant pathogens could have negative consequences for both ecosystem function and human health (Kim *et al*., 2012; Karahan, 2024). Long-term monitoring of microbial community composition in chronically contaminated areas is needed to assess whether these shifts persist and whether they affect nutrient cycling, pathogen resistance, or other ecosystem services. Multigenerational studies tracking both *Artemia* population dynamics and microbial community structure would provide valuable insights into the cumulative impacts of sustained WAF exposure in coastal ecosystems.

CONCLUSION

In conclusion, this study reveals a critical paradox in the toxicological assessment of crude oil WAF: while acute exposure to WAF did not induce observable changes in traditional toxicity endpoints such as mortality, hatching, or morphological malformations in *A. franciscana*, significant molecular disruptions were detected at the genetic level. Most notably, the concentration-dependent inhibition of vital stress-response genes (*P26*, *Hsp26*, *Hsp90*) at low WAF concentrations, contrasted with their upregulation at high concentrations, suggests that the absence of visible acute effects may mask silent cellular damage with potentially severe consequences for long-term population resilience. The downregulation of *P26* and *Hsp90* at environmentally relevant low doses raises particular concern, as these genes are essential for maintaining cryptobiotic survival and cellular homeostasis. Compromised expression of these protective mechanisms may not manifest as immediate mortality but could progressively erode the organism's capacity to withstand subsequent stressors, survive adverse conditions through diapause, or successfully reproduce. Similarly, the selective proliferation of *S. marcescens* at high WAF concentrations demonstrates bioremediation potential, but given its status as an opportunistic pathogen with documented antimicrobial resistance, this species may not be a suitable candidate for environmental bioremediation without carefully managed conditions.

Collectively, these findings position crude oil WAF as a stressor capable of inducing biologically significant effects that remain mostly undetected by conventional acute binary toxicity metrics. This study highlights the need for a more integrative ecotoxicological framework that captures early-warning signals of disruption across different levels of biological organization. Such an approach is essential not only for improving environmental risk assessment, but also for anticipating cascading ecological and potential public health consequences associated with petroleum hydrocarbon exposure.

ACKNOWLEDGMENTS

The authors thank Caio Andrade Medina for the technical support.

AUTHOR CONTRIBUTIONS

CAFS: Conceptualization, investigation, methodology, writing – original draft; **RAL**: Methodology, data curation, investigation, validation; **FDS**: Methodology, Investigation; **RAFS**: Methodology, conceptualization, validation; **ET**: Conceptualization, investigation, methodology, visualization, validation, project administration, supervision, resources

FUNDING

This study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 88887.571446/2020-00 and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) grant number 2022/05498-6.

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