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Inducibility of a Mixed Function Oxidase associated enzyme in the sea anemone *Anthopleura elegantissima* (Cnidaria, Anthozoa, Actiniaria) by Benzo(a)pyrene

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Abstract. Polycyclic aromatic hydrocarbons (PAHs) are a group of environmental contaminants widely studied for their high toxicity in distinct life forms, including humans. Benzo[a]pyrene [B(a)P], one PAHs, is ranked number 8 (of 275 chemicals) on the Priority List of Hazardous substances for humans. In order to understand biotransformation mechanisms of B(a)P, the biochemical response of the sea anemone *Anthopleura elegantissima* to B(a)P was evaluated in the present work. A bioassay was performed using three groups injected with 100 µl of 2-, 4-, and 6-mM B(a)P, and two controls treated with either 100 µl of seawater or solvent (named GC and GS). After 72 h, nuclear, mitochondrial, and microsomal fractions were prepared from the columnar tissue, and NADPH cytochrome C reductase was measured. Enzyme activity in GC organisms was 1.135 ± 0.006, 2.95 ± 0.04, and 8.913±0.13 nmol min-1 mg-1 in nuclear, microsomal, and mitochondrial fractions, respectively, with no difference between control groups. For the B(a)P treated organisms, only the microsomal fraction showed a correlation between the toxic and NADPH cytochrome C reductase. The strong positive correlation between pollutant and the biochemical response point to the inducibility of the enzyme by benzo(a)pyrene in *A. elegantissima.*

Keywords: Oxidase enzyme, *Anthopleura elegantissima*, biotransformation, polycyclic aromatic hydrocarbons.

Inducción de la Oxidasa de Función Mixta con Benzo(a)pireno en la Anémona de Mar *Anthopleura elegantissima* (Cnidaria, Anthozoa, Actinidaria). Resumen: Los hidrocarburos aromáticos policíclicos (PAHs) son contaminantes ambientales ampliamente estudiados por su toxicidad en distintas formas de vida, incluida la humana. El Benzo[a]pireno [B(a)P], un PAHs, ocupa el lugar número 8 (de 275 químicos) en la Lista Prioritaria de Sustancias Peligrosas para humanos. Con el objetivo de entender mecanismos de biotransformación para los B(a)Ps, en el presente proyecto se evaluó la respuesta bioquímica de la anémona de mar *Anthopleura elegantissima* al B(a)P. Se utilizaron tres grupos con 100 µl de B(a)P 2, 4 y 6 mM y, dos controles tratados con agua de mar y disolvente (GC y GS). Después de 72 h se prepararon fracciones nucleares, mitocondriales y microsomales a partir del tejido columnar y se evaluó la actividad de la enzima NADPH citocromo C reductasa. La actividad enzimática en GC fue de

1.135 ± 0.006, 2.95 ± 0.04 y 8.913 ± 0.13 nmol min-1 mg-1 para las fracciones mencionadas, sin diferencias entre controles. Para los organismos tratados con B(a)P, solo la fracción microsomal mostró una correlación entre el tóxico y la NADPH-citocromo C reductasa. La alta correlación positiva entre el contaminante y la respuesta bioquímica indica la inducibilidad de la enzima por benzo(a)pireno en *A. elegantissima.*

Palabras clave: Oxidasa de función mixta, *Anthopleura elegantissima*, biotransformación, hidrocarburos arómaticos policíclicos.

Introduction

Benzo[a]pyrene [B(a)P] is one of the polycyclic aromatic hydrocarbons (PAHs), a class of chemicals identified as a group of widespread environmental contaminants. PAHs form during the incomplete combustion of organic material, and they also occur naturally in coal, crude oil, and gasoline. Due to their toxicity to humans and other forms of life, PAHs are of great concern as a threat to health. B(a)P is ranked number 8 out of 275 chemicals on the Priority List of Hazardous substances, based on the potential for human exposure and the potential health hazard amongst other factors (Risk Information System, 2017).

Marine oil spills have become a health problem for aquatic life in the last decades due to the large amounts of crude oil released into the ocean or coastal areas (Zhang et al., 2018). PAHs are known to be biodegradable; however, Harmsen & Rietra (2018) describe degradation as very low for 5- and 6-ring PAHs. According to Duan *et al*. (2018), PAHs accumulate in marine sediments and remain highly stable even for five years after an incidental oil spill. This long-term stability increases the probability of exposition for the marine ecosystem, and therefore, it sheds a challenge for marine life to counteract the effects of PAHs, namely toxicity, mutagenicity, and teratogenicity. Specifically, B(a)P can cause heritable DNA mutations due to adduct formation between B(a)P molecule and DNA (Moorthy, Chu, & Carlin, 2015).

The marine environment has been widely studied to investigate persistent pollutants' sources and their fate. Sediments, water, and marine life have shown the accumulation, persistence, and effects of B(a)P (Duan et al., 2018; Harmsen & Rietra, 2018; Lawson, Cullen, Nunnally, Rowe, & Hala, 2021; Livingstone, 2001; Overmans et al., 2018; Wolska, Mechlińska, Rogowska, & Namieśnik, 2012). On the contrary, there is scarce research on the effects of B(a)P at the molecular and biochemical level, two types of response that can be used as biomarkers. Also, animal models are needed as bioindicators for environmental monitoring programs. Cytochrome P450s (CYPs) enzymes are mixed-function oxidases (MFO) biomarkers that serve as terminal oxidases in electron transfer chains where they form a complex with a flavoprotein known as NADPH cytochrome P450 reductase. CYPs are valuable biomarkers due to their sensitivity and inducibility upon exposition to PAHs. A high number of CYPs has been reported, but little is known about MFO activity in cnidarians. Further, there is less information about subcellular localization. This enzymatic system has been established in five species of sea anemones and one species of coral (Heffernan & Winston, 1998). The studies that have been carried out in anemones in the field of ecotoxicology are focused on the biotransformation of benzo(a)pyrene [B(a)P]. MFO has been identified in *Hydra littoralis*, where *in vivo* epoxidation was confirmed, presumably due to the activity of MFO (Khan, Kamal, Wolin, & Runnels, 1972). In the Anthozoa, the *in vitro* metabolism of B(a)P was detected in the anemones *Anthopleura xantogrammica* and *Bunodosoma cavernata*, and in the coral *Favia fragum* (Gassman & Kennedy, 1992; Heffernan, Mayeaux, Vasquez, & Winston, 1998).

Sea anemones are simple organisms with anatomy that facilitate the localization and the isolation of subcellular fractions to analyze macromolecules. Anemones can bioaccumulate fossil fuels (Liu et al., 2020), an ability that makes them useful as bioindicators of the presence of B(a)P (Lawson et al., 2021). At the biochemical level, sea anemones are suitable animal models for studying B(a)P effects as enzyme systems related to this xenobiotic have been demonstrated in isolated microsomes. Heffernan & Winston (1998) discovered the presence of Cytochrome P450 and components of mixed-function oxidase in microsomes from the sea anemones *Bunodosoma cavernata*, *Anthopleura elegantissima*, and *Anthopleura xanthogrammica* incubated with B(a)P. Winston, Mayeaux, & Heffernan (1998) proved the inducibility of functioning monooxygenase system

related to several oxidative metabolites by treating microsomes from the columnar region of sea anemone *Bunodosoma cavernata* with B(a)P. Using this xenobiotic, Gómez-Gutiérrez & Guerra-Rivas (2010) performed toxicity tests with *A. elegantissima* and demonstrated the increase of NADPH Cytochrome C reductase in microsomes from the columnar region of the whole organism under B(a)P. The purpose of the present study is to investigate the inducibility of NADPH Cytochrome C reductase in subcellular fractions from the columnar region of whole individuals of *A. elegantissima* exposed to B(a)P.

Material and methods

A. elegantissima: Organisms were collected at Popotla beach in the Municipality of Rosarito, B.C. (32º 17' N; 117º 2' W). The area is exposed to the action of strong waves. Fifty anemones were collected within the intertidal zone. Individuals between 3.0 and 3.6 cm in diameter were selected and placed in coolers with seawater from the site. Once collected, the anemones were transported to the Marine Pharmacology Laboratory of the Facultad de Ciencias Marinas in Universidad Autónoma de Baja California. They were washed, removing sand and pieces of the shell; after, anemones were distributed in glass jars with a capacity of 3 L previously filled with filtered seawater (0.45 mm) with a salinity of 34 ‰. Two anemones of similar size were placed in each flask. The organisms were kept at 17 ºC, with a 12 x 12 photoperiod. All the anemones were fed on the first and third day with pieces of white fish.

Preparation of solutions: Solubility tests were performed for benzo(a)pyrene in solvents: 20% acetone in seawater, 40% acetone in seawater, 60% acetone in seawater, pure acetone, dichloromethane, and acetonitrile. Based on these tests, we decided to use acetone as a solvent to prepare the three toxicant solutions used in the exposition experiment since the highest solubility of B(a)P was in this solvent.

B(a)P exposure: Five groups of 10 organisms were formed. The groups were named according to the concentration of B(a)P to which the organisms were exposed: 2 mM (G2), 4 mM (G4), and 6 mM (G6). In addition, the control group was named GC, made up of organisms to which no addition was made, and GS to the group exposed to the solvent (acetone). Each organism was injected into the trunk area with 100 µl of the corresponding solution. Once injected, the organisms were kept under observation for 72 h. In this period, no water exchanges were made, the 12x12 photoperiod was maintained, and they were not fed. The anemones were not moved from the acclimatization flask, only the water was changed to remove food particles and body excretions. The experiment was terminated after 72 h. At this time, dissection of the columnar tissue of each anemone was taken following ethical guidelines, and all tissues were immediately placed at -80 ºC.

Subcellular fractions preparation: Columnar tissue from organisms under the same exposure conditions was used for each fraction. Tissues were rinsed, weighed, cut into small pieces and then homogenized with five volumes of buffer in an Elvehjem-Potter homogenizer. The homogenization buffer was 0.065 M potassium phosphate pH 7.0, containing 1.15 % KCl and 3 mM MgCl2 (Stegeman, B. Kaplan, & Kaplan, 1981). The subcellular fractions were obtained in stepwise centrifugation, at 750xg for 10 min (nuclear) and 13,000xg for 10 min (mitochondrial) in a Biofuge 22R-Haereaus Instruments ultracentrifuge. The obtained pellets were resuspended in 2 mL of 0.1 M phosphate buffer pH 7.3 for each gram of tissue. For microsomal fraction, tissues were homogenized with four volumes of potassium phosphate buffer 0.1 M pH 7.6 containing 125 mM sucrose (Heffernan et al., 1998). Homogenates were serially centrifuged in a Biofuge 22R (Haereaus Instruments) for 15 min at 8500xg and 15 min at14000xg. The resulting pellets were discarded. Then, the supernatant was centrifuged for one hour at 105000xg in a Sorvall-20/17. The final pellet (microsomes) was resuspended in 0.1 M potassium phosphate pH 7.6, containing 250 mM sucrose, and stored at -80 ºC. This preparation was approximately 4 g of tissue/mL of buffer solution.

Enzyme activity: NADPH cytochrome C reductase activity was measured by following the cytochrome C reduction overtime at 550 nm. The test was carried out according to Heffernan *et al*. (1998). The reaction mixture was: 0.048 mM cytochrome C; 1 mM NADPH, 1 mM KCN; 0.1 M phosphate buffer, pH 7.7, and 0.02 mL of the subcellular fraction. The reaction was started by adding the cofactor NADPH. Enzyme activity was defined as the amount of nmol of cytochrome C reduced per minute per mg of protein (nmol min-1 mg-1 protein). The activity found in the three subcellular fractions of the GC group was used as the basal activity for the GS, G2, G4, and G6 groups. The extinction coefficient to calculate the activities was 21 cm-1 mM-1 (Heffernan et al., 1998). Protein concentration was determined according to the method described by

Lowry, Rosebrough, Farr, & Randall (1951) in samples of 0.030 mL from each homogenate and diluted to 1 mL with water. Protein concentrations were calculated based on the equation obtained by a calibration curve (0-0.5 mg mL-1) prepared with a standard albumin solution (0.5 mg mL-1).

Surrounding seawater released B(a)P evaluation: The seawater from each exposure jar was examined to identify any B(a)P released by the anemones. After the 72-h exposure and removal of organisms, a 100 mL seawater sample was taken from each glass jar and extracted with dichloromethane. This procedure was performed three times, and the extracts pooled together. The solvent was evaporated to dryness, and the residues were resuspended in 2 mL of dichloromethane. The spectrum was run from 200 to 400 nm for each sample, using surrounding seawater from the control group (GC) for the baseline.

Statistical analysis: The Kolmogorov-Smirnov test was applied to verify whether the enzyme activity in each cell fraction at the tested B(a)P concentrations was normally distributed. Homogeneity of variances was analyzed using the Bartlet test. One-way univariate analysis of variance (ANOVA) was used to evaluate the effect of B(a)P on NADPH cytochrome C reductase activity related to the concentration of B(a)P (Zar & Zar Jerrold H, 2010). Differences in enzymatic activity as a function of B(a)P concentration were assessed for nuclear and mitochondrial using the Tukey test. All correlation coefficients (r) (height and weight; B(a)P concentration and enzymatic activity) were calculated with Statistica 5.0 (TIBCO Software Inc).

Results

Organisms: The sizes and weights of the experimental groups did not show significant differences (α = 0.0010), maintaining a general mean size of 3.22 ± 0.19 cm (Table I) in each glass jar. The sizes of the organisms were found between 3.00 and 3.6 cm in the crown and the weights between 12.63 and 31.23 g. A linear correlation was found between the weight and height of the 50 organisms used in the bioassay (r=0.9257)

Surrounding seawater released B(a)P evaluation: There was no B(a)P signal in any of the 15 flasks after the 72 h-exposure. None of the maximum absorbance peaks of benzo (a) pyrene (264, 284, 296, 365, and 383 nm) appear in the surrounding media, according to the spectra in Figure 1. In most of them, negative absorbances were found between 200 and 280 nm.

Enzyme activity: NADPH cytochrome C reductase activity was found in the three subcellular fractions of all experimental groups (GC, GS, G2, G4, G6). We compared NADPH cytochrome C reductase activity in nuclear, mitochondrial, and microsomal fractions with the corresponding fraction in the group to be compared.

Nuclear fraction: The statistical analysis revealed no significant differences between fractions from GC and GS groups. Nuclear fraction enzyme activities ranged from 1.00 to 2.144 nmol cytochrome C reduced min-1 mg-1. G2 and G4 groups showed the highest activities in the nucleus with no significant differences among themselves (α = 0.05). The lowest activities were in the GC, GS, and G6 groups with values 1.135, 1.183, and 1.05 nmol cytochrome C reduced min-1 mg-1 respectively (Fig. 2). There were no significant differences among GC, GS, and G6 (α = 0.05). The basal activity in the mitochondria was higher than the basal activity found in both the nuclear and microsomal fractions. The mitochondrial activities of the NADPH cytochrome C reductase of all the groups were very high, being GC and GS groups the highest, with no significant differences among them (α = 0.05) (Fig. 2). For the nuclear fraction, r = 0.05.

Mitochondrial fraction: The mitochondria activities for the exposed organisms behaved similarly to those found in the nuclear fraction, and no significant differences were observed between the activities of the G2 and G4 groups (α = 0.05). However, in the mitochondria of the G6 group, the enzymatic activity decreased 16.2% with respect to the activity of the G4 group. A negative correlation coefficient was obtained (r = -0.79).

Table I. Average weights and sizes (± d.s.) of *Anthopleura elegantissima* specimens for the five experimental groups used in the bioassay carried out in this work. In all cases, the number of organisms per group (n) was 10. The substances and concentrations to which each group was exposed are shown. B (a) P = Benzo (a) pyrene. Solvent = Acetonitrile.

Group	GC	GS	G2	G4	G6
Medium	Seawater	Solvent in seawater	2 mM B(a)P	4 mM B(a)P	6 mM B(a)P
Size (cm)	3.2 ± 0.12	3.22 ± 0.2	3.22 ±0.22	3.22 ± 0.21	3.22 ± 0.21
Weight (g)	18.94 ± 4.7	18.94 ± 4.30	17.91 ± 6.20	17.81 ± 6.18	18.85 ± 7.23

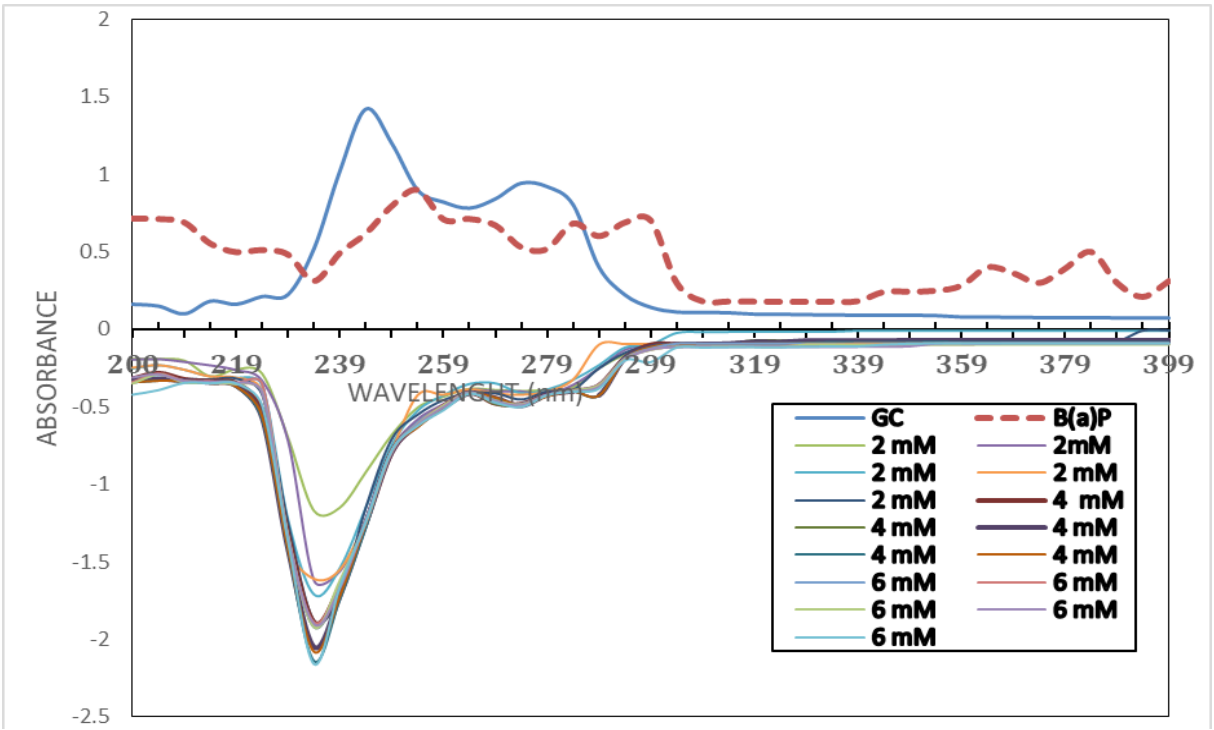

Figure 1. UV absorption spectra of seawater extracts. The extracts were obtained after removing the specimens of *Anthopleura elegantissma* from the jars in the exposure experiment. Seawater from each exposure recipient was treated with dichloromethane and the absorbance of the organic phase was read. Organisms were subjected to B(a)P exposure for 72 h. Concentration values for each group (10 individuals) exposed are indicated. For comparison, B(a)P spectrum is a 56 mM B(a)P in seawater.

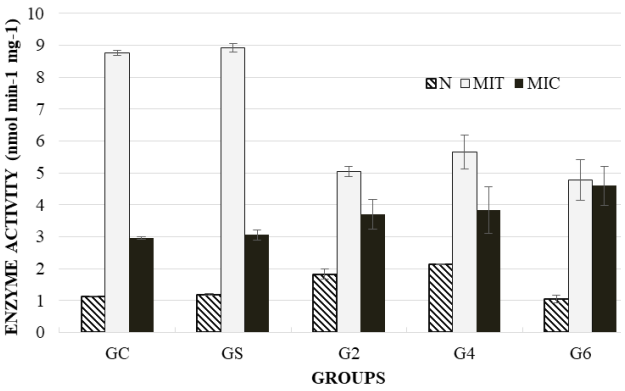

Figure 2. NADPH cytochrome C reductase activity in subcellular fractions of columnar tissue from sea anemone Anthopleura elegantissima under exposure to benzo(a)pyrene for 72 h. In all cases, the number of organisms per group was 10. Values are the average of five measurements. The enzyme activity is expressed in nanomoles of reduced cytochrome C per minute per milligram of protein. B (a) P = Benzo (a) pyrene. Solvent = Acetonitrile. GC = Control group; GS = Solvent group; G2, G4, and G6 = Groups exposed to 2 mM, 4 mM, and 6 mM B(a)P respectively. N = Nuclear fraction MIT = Mitochondrial fraction.

Microsomal fraction: The GC and GS group activity was 2.95 ± 0.04 and 3.05 ± 0.157 nmol cytochrome C reduced min-1 mg-1 protein, respectively. In this fraction, as in nuclei and mitochondria, no significant differences were found between the activities of these two groups (α = 0.05). In the organisms exposed; we observed increments in enzyme activity related to the B(a)P concentration increment. In microsomal fraction, NADPH cytochrome C reductase was 25, 30 and 80 % more active than controls in the organisms exposed to 2 mM, 4 mM, and 6 mM, respectively. All these activities were calculated with respect to the basal activity obtained for the organisms control group (2.9504 ± 0.04). For this fraction, a high correlation was obtained (r = 0.98) between NADPH cytochrome C reductase activity and the concentration of B(a)P.

Discussion

Polycyclic aromatic hydrocarbon contaminants have been a cause for concern over many decades worldwide. Recent studies showed evidence for fossil-fuel particulate matter with a diameter of approximately 1µm (PM1) accumulation

in marine biota (Liu et al., 2020). According to Chimjarn, Delhomme, & Millet (2021), PM1 is related to high molecular weight (HMW) (5-ring and 6-ring) PAHs. As reported by Liu *et al*. (2020), six species of the genus *Anthopleura*, *Actinia equina*, *A. asiatica*, *Diadumena lineata*, *Stylobates aeneus*, and *Boloceroides mcmurrichi* are capable of bioaccumulating PM1, reaching 5-7 times their surrounding seawater concentration. The finding of PM1 in sea anemone eggs and brooding and released juveniles sends a warning signal for a possible maternal transfer of the contaminant. Undoubtedly, there is still an urgent need to develop and implement more and better monitor environmental programs.

Besides directly measuring the pollutant, biomarkers and bioindicators are excellent tools to approach pollution studies. The discovery of enzymes related to cytochrome P450 in sea anemones (Heffernan & Winston, 1998) showed the possibility for these organisms to be bioindicators of PAHs in marine environments. This finding also suggests that enzymes associated with cytochrome P450 could be practical PAH biomarkers for coastal marine pollution research. In our study, one of those enzymes, NADPH cytochrome C reductase was found in all the fractions obtained from the columnar tissue. However, our results show B(a)P different intervals of activity on the nucleus, mitochondria, and microsomes of *A. elegantissima*. NADPH cytochrome C reductase activity found in the control group (CG) was the lowest. In general, for the GC group, the values were: nucleus < microsomes < mitochondria. Our results for the nuclear fraction are comparable with those found in the barnacle *Balanus ebemeus*, reported by Stegeman *et al*. (1981). These investigators reported activity of 0.819 nmol reduced cytochrome C min-1 mg-1 protein. For the marine fish *Stenotomus versicolor* the activity in nuclei was established as 0.219 nmol cytochrome C reduced min-1 mg-1 protein (Stegeman & Binder, 1979).

The microsomal fraction has been the most studied for identifying MFO since the NADPH cytochrome C reductase activity is high within the endoplasmic reticulum (Solé & Livingstone, 2005). The baseline value for microsomes determined in the present study (2.961 ± 0.19 nmol reduced cytochrome C min-1 mg-1 protein) is within the interval reported for cnidarians by Heffernan & Winston (1998), who found values of 3.4 ± 0.5, 2.0 ± 0.2, and 2.1 ± 0.3 nmol cytochrome C reduced min-1 mg-1 protein for the microsomes of *A. elegantissima*, *A. xantogrammica* and *Bunodosoma cavernata* respectively. These data place cnidarians in the lowest part of the values obtained for invertebrates since the range of NADPH cytochrome C reductase activity for most invertebrates is reported between 2 and 10 nmol min-1 mg-1. The closest activities are those in the hedgehog *Echinus esculentus* (5.6 ± 1.9 nmol min-1mg-1), although higher values have been reported for other echinoderms (Den Besten, 1998). Activities higher than 10 nanomole-1 min-1 mg-1 have been found in organisms such as the barnacle *Balanus eberneus* (Stegeman et al., 1981) and the mussel *Mytilus edulis* (Livingstone, Moore, Lowe, Nasci, & Farrar, 1985). These high values should be expected, given that barnacles and bivalve mollusks have specialized organs where NADPH cytochrome C reductase must be active. On the contrary, Cnidarians have less evolutionary development with low specialization.

The basal activity found in the mitochondrial fraction was higher than the basal activity in nuclei and microsomes. This result could be explained by the presence of not-removed lipid-containing tissue, adding lipidic molecules to the lacking-fat columnar tissue. The fatty layer seen after the first centrifugation in the samples from the GC and GS group suggests the presence of fatty acids. Also, NADPH could have been present due to its role as a cofactor necessary for fatty acid synthesis. On the other hand, high activities could be expected in the mitochondria since NADH is in high amounts in these organelles because of the Krebs cycle. This nucleotide, like NADPH, can act as an electron donor and, therefore, increase the enzyme activity. To avoid interference of NADH on NADPH function, KCN is added in the enzymatic assay to inhibit the supply of NADH by electronic transport (ATP synthesis), specifically in the complex of cytochrome C oxidase. However, the high levels of NADH could exceed the action of the inhibitor, causing an elevated enzyme activity value.

Regarding the organisms exposed to B(a)P, the nuclear fraction presented an increase of 71.16% in the group exposed to a concentration of 2 mM. This activity is not significantly different from that found in organisms exposed to 4 mM B(a)P. In the 6mM B(a)P group (G6), the activity decreased with no significant differences between this group and the control group. This decrease gives r = 0.06 between the enzyme activity and the B(a)P concentration. Therefore, NADPH cytochrome C reductase is independent of the concentration of the toxic. It is

worth mentioning that nuclear fraction contains cellular elements sedimenting at the same speed as nuclei, such as whole cells, cytoskeletons, and plasma membranes. Due to the presence of these components, we might say the cell nucleus is not practical in the search for biomarkers.

For the sea anemones under B(a)P, we found the highest activity in mitochondrial fractions from organisms exposed to concentrations 2- and 4- mM B(a)P, without significant difference between them (a = 0.05). On the contrary, animals under 6 mM B(a)P showed decreased enzyme activity concerning G2 and G4 groups concentrations. A comparison between GC and GS groups was difficult due to the anomalies found in the latter. Assuming a proportional overestimation in the enzymatic activity from G2, G4, and G6 groups mitochondrial fractions, we found a linear correlation between B(a)P concentration and NADPH cytochrome C reductase activity (r = -0.78). The correlation coefficient also indicates cytochrome P450 does not increase its activity in the presence of the xenobiotic used in the bioassay. This negative value implies the degradation function is not fulfilled, and biotransformation of B(a)P is not warranted in this cellular fraction at a high concentration.

A high correlation was observed in microsomal fraction between NADPH cytochrome C reductase activity and the concentration of PAH (r = 0.961). It appears that microsomal stimulation occurs, favoring a higher activity of the MFO. This fact does not ensure the complete biotransformation of B(a)P in the anemone by the same routes proposed for other marine organisms (Jørgensen, Giessing, Rasmussen, & Andersen, 2008; Mitchelmore, Birmelin, Chipman, & Livingstone, 1998), but the correlation indicates the inducibility of the MFO-associated reductase system. Increases in the activity of reductases associated with MFO and the enzymes involved in phase II of biotransformation have also been found for other marine species exposed to oil, HPAHs, and PAHs. Effects of this type have been reported for marine polychaetes (Lee, Stolzenbach, Singer, & Tenore, 1981), mesopelagic fishes (Bozinovic et al., 2021; García-Segura et al., 2018), periwinkles, mussels, and clams (Livingstone et al., 1985; Solé & Livingstone, 2005).

MFO participates in xenobiotic biotransformation and takes part in development processes such as hormone production. For crabs, variations in the MFO-dependent enzymes have been found related to the developmental stage of the organisms (Rewitz, Styrishave, & Andersen, 2003; Singh, Alkins-Koo, Rostant, & Mohammed, 2021). Despite being at a lower evolutionary level, we assumed possible activity fluctuations due to this factor. We collected organisms of similar crown diameters and measured the weight after water expulsion. The correlation coefficient (r = 0.9257) shows a linear increase of the weight as a function of size. Therefore, the size of the organisms used in our experiment is correctly estimated. No significant differences were observed (a = 0.001) between the mean size values of the experimental groups; thus, the size of the organisms does not affect the activity of the NADPH cytochrome C reductase.

Under stressors, sea anemones make body contractions that might expel the toxic into the surroundings. In a bioassay, a loss of B(a)P by organisms must be either ruled out or apply a factor correction. For this reason, we explored the exposure flasks searching for the hydrocarbon in the seawater from the containers. None of the UV spectra (Fig.1) showed maximum absorbance peaks of B(a)P (Chen & Chen, 2001). All the exposure flasks, except for one from G4, showed negative absorbances in the range of 200 to 280 nm. The blank was water from the containers of the GC group, which presented maximum absorbances at 234 and 272 nm. Therefore, when the blank was subtracted, negative absorbances were obtained. In the spectrum with differences of maximum absorbance at 244 nm and 272 nm, there is no agreement with the maxima of B(a)P. These absorbances might be from metabolic waste from anemones. Based on the spectra, there was no residual B(a)P in any of the exposure flasks, thus excluding the expulsion.

Conclusion

In the present work, NADPH cytochrome C reductase, specific for P450, was identified in the three subcellular fractions studied. This finding confirms the enzyme complex MFO is present in the columnar region of *A. elegantissima* (Heffernan & Winston, 1998). Our previous work showed B(a)P induces an increase of the reductase in animals injected with increasing doses of the pollutant (Gómez-Gutiérrez & Guerra-Rivas, 2010). We further report in the present work the presence of this enzymatic system in the nucleus, mitochondria, and microsomes from organisms administered increasing B(a)P concentrations. Together, these facts imply that *A. elegantissima* can respond to

organic xenobiotics from its environment. The high correlation between the pollutant and the effect observed only in the microsomal fraction suggests that this is the only one with a functional MFO-dependent cytochrome P450. Therefore, the microsomal fraction is potentially useful as a sensitive biomarker of PAHs on anemones. The abundance, size, mobility, and morphological simplicity of sea anemones within their niche make them a suitable bioindicator for environmental monitoring programs.

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