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# Acute and Sub-Acute Toxicity of the Polycyclic Aromatic Hydrocarbon 1-Methylnaphthalene to the Shallow-Water Coral *Porites divaricata*: Application of a Novel Exposure Protocol

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**ACUTE AND SUB-ACUTE TOXICITY OF THE POLYCYCLIC AROMATIC  
HYDROCARBON 1-METHYLNAPHTHALENE TO THE SHALLOW-WATER CORAL  
*PORITES DIVARICATA*: APPLICATION OF A NOVEL EXPOSURE PROTOCOL**

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ACUTE AND SUB-ACUTE TOXICITY OF THE POLYCYCLIC AROMATIC HYDROCARBON 1-METHYLNAPHTHALENE TO THE SHALLOW-WATER CORAL *PORITES DIVARICATA*:  
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Running title: Toxicity of 1-methylnaphthalene to *Porites divaricata*

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**Abstract:** Previous research evaluating hydrocarbon toxicity to corals and coral reefs has generally focused on community level effects, and results are often not comparable between studies due to variability in hydrocarbon exposure characterization and evaluation of coral health and mortality during exposure. Toxicity of the polycyclic aromatic hydrocarbon 1-methylnaphthalene to the coral *Porites divaricata* was assessed in a constant exposure toxicity test utilizing a novel toxicity testing protocol uniquely applicable to shallow-water corals, which considered multiple assessment metrics and evaluated the potential for post-exposure mortality and/or recovery. Acute and sub-acute effects (gross morphological changes, photosynthetic efficiency, mortality, and histologic cellular changes) were evaluated during pre-exposure (4 wk), exposure (48 h) and post-exposure recovery (4 wk) periods. Coral condition scores were used to determine a 48 h EC50 of 7,442 µg/L. Significant physical and histological changes were caused by 640 and 5,427 µg/L 1-methylnaphthalene after exposure, with a 1 to 3 d delay in photosynthetic efficiency effects ( $\Delta F/F_m$ ). Pigmented granular amoebocyte area was found to be a potentially useful sub-lethal endpoint for this species. Coral mortality was used to estimate a 48 h LC50 of 12,123 µg/L. This article is protected by copyright. All rights reserved

**Keywords:** Corals; Marine toxicity tests; Polycyclic aromatic hydrocarbons (PAHs); 1-methylnaphthalene; Passive dosing

## INTRODUCTION

As one of the few productive ecosystems that thrive within oligotrophic seas, coral reefs are diverse and complex marine communities which are an essential part of the geology and ecology of tropical and subtropical oceans [1]. The complex communities associated with coral reefs depend on the structural role provided by hermatypic corals [1, 2]. Coral reefs typically exist in coastal environments often directly adjacent to areas of dense human population, providing ample opportunity for anthropogenic impacts (including oil pollution) to have substantial negative effects. Despite multiple studies on the effects of petroleum hydrocarbons on corals, data remain limited compared to other species; few reports from past oil spills refer specifically to corals, and previous research tends to be general and sometimes contradictory [2, 3]. A wide variety of lethal and sub-lethal effects of oil on corals and endosymbionts have been reported, from increased mortality to altered cellular physiological condition in the coral animal [4-6]. Physical changes to corals include tissue contraction, distension and rupture, mesenterial filament extrusion, nematocyst discharge and fragmentation, tentacle retraction, and abnormal polyp behavior with inhibition of feeding or tactile response [4, 5, 7, 8]. Metabolic changes (reduced growth rate, increased protein to lipid ratios, and shifts from metabolic homeostasis), decreased photosynthetic yield and symbiont density have also been noted [4, 7, 9]. When data sets do exist, and effects can be quantified, comparability between effects is usually difficult due to variability in oil composition, different weathering processes, different methods of solution preparation, various exposure conditions, and a lack of quantitative hydrocarbon chemistry of test solutions [3, 10, 11]. Thus, a substantial data gap exists on the toxicity thresholds of hydrocarbons to corals, from the organismal to cellular level.

Crude oil is a complex mixture of several thousand molecular compounds, with each oil containing widely varying amounts of chemicals. The relative solubility and persistence of constituent aromatic hydrocarbons results in crude oils with different toxic impacts [11-13]. A central issue in

toxicity studies is often the lack of quantitative chemical analyses [3, 14, 15], with results frequently based on nominal concentrations; of published studies on hydrocarbon toxicity to corals, only

approximately 21% include quantitative hydrocarbon chemistry. Mixing energy and loading method can have a profound effect on the dissolution, bioavailability, and relative concentration of constituent hydrocarbons [16]. Speciated hydrocarbon characterization is thus necessary for results to be extrapolated to oil spills or compared between studies [10, 11, 15].

An alternative to whole oils in toxicity studies is the use of individual hydrocarbons. Petrogenic hydrocarbons are type I narcotic chemicals with a single toxic mode of action (narcosis). Therefore, the toxicity of specific oils results from only the additive toxicity of constituent hydrocarbons, especially aromatics [12, 13, 17, 18]. The target lipid model provides a quantitative framework for describing the toxicity of dissolved hydrocarbons and is based on the hypothesis that toxicity results when organismal tissue lipid concentrations of a specific hydrocarbon exceed the critical threshold for the organism in question, leading to morbidity and eventual mortality [18]. The hydrophobicity of polycyclic aromatic hydrocarbons (PAHs) promotes partitioning across permeable membranes into organismal tissue lipids until equilibrium is reached [12]. This is particularly relevant to coral tissue which has a high lipid content [19], although uptake and persistence of hydrocarbons during and after exposure may depend significantly on type and duration of exposure, and specific characteristics of the exposed species. Even low concentrations may impair behavioral and developmental processes by disrupting energetic and biosynthetic pathways at the cellular level. Sub-lethal changes to these pathways may result in impairment of feeding mechanisms, growth and development rates, energetics, reproductive outputs, recruitment rates, or other histopathological disorders [2].

Modeling the toxicity of individual hydrocarbons based on lethality and sub-lethal effects permits prediction of the toxicity of any complex hydrocarbon mixture [13, 17], while limiting experimental and analytical challenges. Single hydrocarbons, such as naphthalene, are often a substantial contributor to the PAH content of water-accommodated fractions (WAF) of petroleum



substances, and are therefore commonly used in toxicological studies [20-23]. Alkylated PAHs are usually more abundant than parent PAHs [12, 21, 24, 25], and demonstrate increased toxicity as a result of increased lipophilicity [21, 25]. Consequently, alkylated derivatives such as 1-methylnaphthalene may be more useful than the parent PAH in toxicity studies.

As past studies have used a wide variety of metrics to evaluate coral response to hydrocarbon exposure, and acute mortality can be difficult to assess in benthic sessile organisms such as corals, the present study developed a novel toxicity testing protocol uniquely applicable to shallow-water corals, which considers specific assessment metrics and evaluates the potential for post-exposure mortality and/or recovery. Using this protocol, acute and sub-acute effects (mortality, gross morphological changes, photosynthetic efficiency, and histologic cellular changes) of 1-methylnaphthalene to the shallow-water coral *Porites divaricata* were evaluated during pre-exposure (4 wk), exposure (48 h constant exposure) and post-exposure recovery (4 wk) periods. This research provides new data on sub-lethal and lethal toxicity thresholds of 1-methylnaphthalene to a model coral species.

## **MATERIALS AND METHODS**

### *Pre-exposure (4 wk)*

Branch tips (2 cm in length) of the thin finger coral *Porites divaricata* were collected from shallow waters offshore of Broward County, Florida. This coral was selected due to its growth form and adaptability to laboratory conditions, which make it an ideal model species. The coral fragments were attached with a minimal amount of cyanoacrylate gel glue to small numbered aragonite bases and allowed to acclimate to laboratory conditions in a 300 gallon indoor system. Natural seawater was used; the system was maintained at 26°C and light was provided by LED lights (Photon) (12 h photoperiod, programmed sunrise and sunset). Corals were not fed during the pre-exposure period. The condition of each coral was semi-quantitatively scored [including color, polyp extension/retraction, tissue swelling/distension, and mucus production, on a scale of 0 (normal limits) to 3 (severely affected)]. This scoring system was adapted from a histologically verified stress index developed for

real-time coral health assessment [26]. Photosynthetic efficiency measurements were used as an indicator of the physiological status of the autotrophic endosymbiotic zooxanthellae. The light adapted effective quantum yield  $[(F_m - F)/F_m$  or  $\Delta F/F_m]$  of the endosymbiotic zooxanthellae was determined from the ratio of initial fluorescence ( $F$ ) to maximum fluorescence ( $F_m$ ) by applying a saturation pulse of light using a pulse amplitude modulated fluorometer (Diving-PAM, Walz, Germany). The following parameters were chosen to determine yield for *P. divaricata*: measuring light intensity = 3, damping = 2, gain = 3, saturation intensity = 7, and saturation width = 0.8. These were determined by a combination of published literature values [9], and parameter adjustment until the saturation curve had the characteristic plateau required for accurate depiction of effective quantum yield. Ten randomly selected coral fragments were collected at the end of the pre-exposure period for histological analysis.

#### *Exposure (48 h)*

Coral exposure to 1-methylnaphthalene was conducted using a continuous flow recirculating passive dosing system [13, 27] (Figure 1) in a 48 h constant exposure using chambers similar to those described and employed by the Chemical Response to Oil Spills Ecological Effect Research Forum (CROSERF) [16]. Five treatments were used, with 3 replicate dosing systems per treatment, based on polydimethylsiloxane (PDMS) O-rings as dosing mechanisms. A seawater control (with O-rings), a methanol (MeOH) control (with O-rings), and 3 concentrations of 1-methylnaphthalene were tested (nominally 500  $\mu\text{g/L}$ , 5,000  $\mu\text{g/L}$ , and 25,000  $\mu\text{g/L}$ ). The seawater control was utilized to rule out any effect of the O-rings, and possible effects of the chamber system. The methanol control was used to determine whether a solvent effect resulted from loading of the O-rings. Treatments were randomly assigned to dosing systems.

Before the start of the exposure period, PDMS O-rings (O-Rings West) were cleaned by rinsing in ethyl acetate (Fisher Scientific) (24 h), methanol (Fisher Scientific) (3x in 24 h), and deionized water (3x in 24 h), then dried at 110°C for one h. Stock solutions of 1-methylnaphthalene (Acros Organics, 97%) in methanol were prepared using the equation

$$C_{\text{MeOH}} = \left[ K_{\text{MeOH-PDMS}} + \left[ \frac{V_{\text{PDMS}}}{V_{\text{MeOH}}} \right] \right] \times \left[ K_{\text{PDMS-Water}} + \left[ \frac{V_{\text{Water}}}{V_{\text{PDMS}}} \right] \right] \times C_{\text{Target}}$$

where  $C_{\text{MeOH}}$  is the concentration of 1-methylnaphthalene added to methanol (mg/L);  $C_{\text{target}}$  is the target concentration in seawater (mg/L);  $V_{\text{methanol}}$  is the volume of the methanol dosing solution (mL);  $V_{\text{PDMS}}$  is the volume of PDMS O-rings in the mixing vessel (mL);  $V_{\text{water}}$  is the volume of water in the recirculating flow-through system (mL);  $K_{\text{MeOH-PDMS}}$  is the partition coefficient of 1-methylnaphthalene between methanol and PDMS ( $\log K_{\text{MeOH-PDMS}} = 0.70$ ); and  $K_{\text{PDMS-Water}}$  is the partition coefficient of 1-methylnaphthalene between PDMS and water ( $\log K_{\text{PDMS-Water}} = 2.98$ ) [27].

The calculated amount of 1-methylnaphthalene required for each experimental concentration was dissolved in methanol and mixed for 24 h. Cleaned PDMS O-rings (114 for each concentration/treatment, 38 per replicate, mean mass 1.06g) were added to the methanol stock solutions and allowed 72 h (on an orbital shaker) for adequate partitioning of 1-methylnaphthalene into the O-rings [13, 27]. Calculated depletion of 1-methylnaphthalene in both reservoirs was 4.42% in the MeOH loading solution, and 7.41% in the PDMS O-rings.

Prepared O-rings were then transferred to the assigned dosing systems. Each dosing chamber was filled with 500 mL seawater from the laboratory system, filtered to 1  $\mu\text{m}$  (Polymicro) and 3 O-rings; each dosing vessel was filled with 2300 mL filtered seawater and 35 O-rings (dosing systems had <10% headspace when filled and operational, to limit volatile loss, and were vigorously stirred throughout). The peristaltic pumps were started and the systems were allowed 16 h for equilibration [13, 27].

After equilibration, 5 randomly assigned corals were added to each chamber, and the 48 h exposure was initiated. All equipment was monitored for continuous operation within designated limits throughout the duration of exposure. As during the pre-exposure period, corals were not fed and

lighting was provided by LED lights (Photon) (12 h photoperiod, programmed sunrise and sunset).

Coral condition was assessed hourly for the first 8 h after exposure initiation, and every 12 h thereafter

for the remainder of the 48 h exposure. Semi-quantitative measurements of coral condition were

recorded and percent recent mortality [28] was visually estimated concurrent with coral condition

observations. After the 48 h exposure, the chambers were opened and surviving corals were transferred

back to the laboratory system for monitoring during a post-exposure recovery period. Two randomly

selected corals from each chamber were collected at this time for histological analysis.

#### *Post-exposure recovery (4 wk)*

Following the 48 h exposure, three surviving corals were transferred back to the acclimation

system for the 4 wk post-exposure recovery period. Coral fragments were maintained under the same

conditions as described for pre-exposure. Corals were not fed during the post-exposure period.

Condition of each coral was assessed daily for 1 wk, and twice weekly thereafter, using PAM

fluorometry and semi-quantitative measurements of coral condition and mortality as previously

described. All remaining coral fragments were collected for histological analysis at the end of the post-

exposure period.

#### *Histology*

Coral samples for histological analysis were fixed in 2% glutaraldehyde in sodium cacodylate

buffered seawater for 4 d to 6 d at 4°C, then decalcified in 5% HCl/EDTA seawater solution,

dehydrated in a graded series of ethanols and xylene, and embedded in paraffin wax (Paraplast Plus).

Longitudinal and transverse sections (4 µm) were mounted on slides. Sections were cleared in xylene

and stained with Hematoxylin & Eosin. Stained slides were viewed in an Olympus BX 43 light

microscope at magnifications ranging from 4x to 60x and photographed with an Olympus DP21 digital

camera for image analysis of cellular structures. Coral tissues were assessed for quantitative changes in

overall tissue characteristics, individual cell types and degeneration of tissues. Digital micrographs

were calibrated in Image J, and tissue and cellular characteristics were measured on screen. Area of the

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epidermis and gastrodermis in the coenenchyme (the common mesenchymal tissue which links colonial polyps in corals) was determined by tracing the edge of each layer along a 100  $\mu\text{m}$  contour length (ten per sample), and the area of mucocytes and granular amoebocyte cells was determined by tracing the cell margins. These measurements were then used to quantify the relative surface area per contour length for each metric.

#### *Hydrocarbon chemistry and water quality*

Water samples for 1-methylnaphthalene analysis were collected from a port on the outflow line of each chamber (Figure 1). Samples were collected in certified volatile organic analyte vials (Thermo Scientific) vials (with no headspace) at the start (0 h, immediately prior to addition of corals), middle (24 h), and end (48 h, immediately prior to removal of corals) of the exposure to verify the stability of the concentration throughout the exposure. Five duplicate samples were collected and analyzed at each time point. Samples were preserved at 4°C and concentration of 1-methylnaphthalene was quantified in a Horiba Aqualog Spectrofluorometer after extraction with dichloromethane (Sigma Aldrich). All solvents used in these analyses were analytical grade and tested prior to use. A calibration curve with at least five points was run daily (analytical standard, Supelco); the coefficient of determination ( $r^2$ ) was required to be greater than 0.99 before the samples were run. Blanks were run vs air and tested to determine that no emission was observed at the wavelengths (excitation and emission) used for 1-methylnaphthalene.

Additional water samples for basic water quality were collected at the start and end of the exposure. Nutrients [ammonia ( $\text{NH}_3$ ), nitrite ( $\text{NO}_2$ ), nitrate ( $\text{NO}_3$ ), phosphate ( $\text{PO}_4$ )] were measured with a HACH DR850 colorimeter; pH, dissolved oxygen (DO) and temperature were measured with a YSI 556 Multiprobe System; and alkalinity was determined by potentiometric titration with a Mettler-Toledo DL22 autotitrator.

#### *Statistical analyses*

All data were tested for normality (Brown-Forsythe) and homoscedasticity (Kolmogorov-Smirnov/Lilliefors) and transformed to meet these assumptions where applicable, or nonparametric methods were used. Tukey's Unequal N HSD (parametric) or Multiple Comparisons (nonparametric) was used for post-hoc analysis. All statistical tests were performed using STATISTICA 12.

Kruskal Wallis ANOVA on ranks ( $\alpha=0.05$ ) with untransformed data was used to compare mean coral condition score (mean of 5 coral fragments in each replicate,  $n=3$  replicates) between treatments (during pre-exposure, exposure, and post-exposure periods) and water quality data between treatments. One-way ANOVA on untransformed data was used to compare mean quantum yield (mean of 5 coral fragments in each replicate,  $n=3$  replicates) between treatments over the pre-exposure and post-exposure periods. To compare histological characteristics, mixed-model 3-way nested ANOVA [Factors: Treatment, Chamber and Coral (Chamber)] ( $\alpha=0.05$ ) was used to compare mean area (mean of 10 measurements per coral,  $n=2$  corals per time point) between treatments at each time interval.

The median lethal concentration (LC50) was calculated using the graphical method [29]. The median effect concentration (EC50) was calculated from mean coral condition scores with GraphPad Prism 6.0.

## RESULTS AND DISCUSSION

### *Hydrocarbon chemistry and water quality*

Measured concentrations of 1-methylnaphthalene over the exposure period for each treatment are shown in Table 1 (and Supplemental Data, Table S1). All of the aqueous concentrations were in general agreement with predicted values, with a maximum mean variability in concentration of 13.2%, and a maximum mean loss of 5.79% over 48 h for all of the exposure levels. The variability in concentration between replicates likely resulted from free material adsorbed to the O-rings; additional rinsing of O-rings before transfer to the dosing system has been subsequently added to the methodology.

Similar to previous experiments utilizing passive dosing [13], the present study demonstrates the value of this methodology in achieving and maintaining relatively stable PAH concentrations during dosing experiments with minor loss over test periods. Most petroleum PAHs are sparingly soluble, and obtaining constant exposure concentrations can be challenging due to loss mechanisms (sorption, volatilization, and degradation) [13, 30]. Studies without passive dosing often demonstrate substantial declines in PAH concentration over the exposure period; for example, a 35–55% loss of total PAH over 24 h in exposure vessels with >10% headspace [20], a 64% decline in total PAH over 84 h [31], and 20.9% and 10.8% loss over 24 h of naphthalene and 2-methylnaphthalene, respectively [32]. Although naphthalene has a relatively high water solubility compared to other PAHs, loss in the exposure system will occur without a reservoir to equilibrate at a specified concentration.

#### *Quality assurance and quality control*

Coral condition and photosynthetic efficiency were consistent in all corals during the pre-exposure period; no significant differences in mean coral condition score ( $p=0.4159$ ) or mean quantum yield ( $F_{(5,12)}=1.532$ ,  $p=0.2518$ ) were found. Histological analysis indicated no significant differences in mean epidermal mucus area %, gastrodermal mucus area %, epidermal pigmented granular amoebocyte area % or gastrodermal pigmented granular amoebocyte area % between corals collected at the end of the pre-exposure period and corals from both control treatments after the exposure period ( $p>0.05$ ). Additionally, no significant effect was found for the nested random factor of treatment chamber ( $p>0.05$ ) in the analysis of histological characteristics after the exposure period.

A summary of water quality parameters is found in Supplemental Data, Table S2. Significant increases ( $p<0.05$ ) in nutrient concentrations ( $\text{PO}_4$ ,  $\text{NH}_3$ , and  $\text{NO}_2$ ) and significant decreases ( $p<0.05$ ) in pH and DO were found in the 25,832  $\mu\text{g/L}$  treatment chambers compared to other treatments, likely due to coral tissue necrosis in the highest concentration tested. No significant differences ( $p>0.05$ ) in pH, alkalinity,  $\text{PO}_4$ ,  $\text{NH}_3$ ,  $\text{NO}_2$ ,  $\text{NO}_3$  or DO were found between the seawater control, MeOH control,

640 µg/L or 5,427 µg/L treatments, and no significant difference ( $p>0.05$ ) in temperature was found between all treatments.

#### ● *Physical coral response, LC50 and EC50*

Progressive coral physical response is shown in Figure 2. Overall, corals in both the seawater and methanol control treatments exhibited normal polyp extension, with limited mucus production and no tissue swelling during the 48 h exposure period. Corals exposed to 640 µg/L displayed some polyp and coenenchyme distension as well as a qualitative delay in tactile response after 48 h; both are consistent with a narcotic action and have been observed in other coral species in response to oil and drilling mud exposure [33]. The 5,427 µg/L exposed corals had marked, progressive polyp retraction, moderate tissue swelling and mucus production after 24 h. The corals exposed to 25,832 µg/L exhibited full polyp retraction and substantial mucus production within 6 h of exposure, with 100% mortality occurring after 24 h. Mesenterial filament extrusion was not apparent, although this is noted as a response to hydrocarbon exposure in other coral species [33]. As no coral mortality was found at a concentration of 5,427 µg/L and no partial mortality was observed, the graphical method was used to calculate an LC50 of 12,123 µg/L.

The highest mean concentration tested, 25,832 µg/L, resulted in a 600% increase in condition score after 1 h of exposure, and 5,427 µg/L resulted in an overall 681% higher coral condition score compared to both control treatments after 48 h. The lowest exposure concentration, 640 µg/L, did not result in significant changes to condition score compared to both control treatments.

Comparison of mean coral condition score for each treatment at each interval over the pre-exposure, exposure, and post-exposure periods found significant treatment effects at all time points from 1 h after initiation of exposure to 9 d post-exposure ( $p<0.05$ ). Post-hoc analysis indicated that the 5,427 µg/L and 25,832 µg/L treatment corals scored significantly higher than the 640 µg/L and both control treatments at the end of the exposure period (Figure 3A). After 1 d of recovery, the 640 µg/L corals scored similarly to both control treatments ( $p>0.05$ ) while the 5,427 µg/L coral scores remained



significantly higher ( $p < 0.05$ ) than both control treatments until 1 wk of recovery (Figure 3A). After 9 d of recovery no treatment effect on coral condition was observed ( $p > 0.05$ ). Coral condition scores were used to calculate an EC50 of 7,442  $\mu\text{g/L}$  (95% CI: 4,905–11,290  $\mu\text{g/L}$ ).

Mean quantum yield (Figure 3B) was not significantly different between treatments at the end of the pre-exposure and exposure periods, or after 1 wk of recovery ( $p > 0.05$ ). However, mean quantum yield of the 5,427  $\mu\text{g/L}$  corals was significantly higher than both control treatments from 1 to 3 d post-exposure, and higher than the 640  $\mu\text{g/L}$  corals from 1 to 4 d post-exposure ( $p < 0.05$ ) (data not shown). After 1 wk of recovery, no significant differences between treatments were found ( $p > 0.05$ ) (Figure 3B).

The observed increase in photosynthetic efficiency in the 5,427  $\mu\text{g/L}$  exposed corals is in contrast to other studies [9], but may be related to the increase in granular amoebocytes in the epidermis or be driven by increased demand, as zooxanthellae are involved in mucus production [1].

### *Histology*

Histologically, control corals had normal cellular architecture, with columnar epidermis, intact mucocytes and abundant granular amoebocytes in the coenenchyme (Figure 4A). After 48 h of exposure to 640  $\mu\text{g/L}$ , some tissue swelling was evident, concomitant with elevated mucus production in the epidermis (Figure 4B). After 48 h of exposure to 5,427  $\mu\text{g/L}$ , epidermal structure was compromised with atrophy of epidermal mucocytes and extensive swelling of the gastrodermis (Figure 4C). The coral surface mucus layer, as the interface between the coral epithelium and the environment, is of central importance as a primary protective physiochemical barrier and plays a central role in ciliary-mucus feeding and surface cleansing [34]. It has been suggested that mucus may bind or absorb pollutants such as aromatic hydrocarbons [24] or metals [35, 36] and so confer some protection to the underlying coral tissues either by providing a physical barrier or as an avenue for pollutant release [24]. Increased mucus secretion, hyperplasia, hypertrophy, and atrophy of mucocytes has been described as a response to oil exposure in several other coral species [7, 24]. In the present study, exposure to 25,832  $\mu\text{g/L}$  resulted in marked, significant mucus secretion within 4 h of exposure. Significant treatment

effects were found for both epidermal mucocyte area ( $F_{(3,8)}=13.762, p=0.0016$ ) and gastrodermal mucocyte area ( $F_{(3,8)}=5.277, p=0.0267$ ) after 48 h of exposure. No significant differences in mucocyte area were found after the post-exposure recovery period ( $p>0.05$ ).

Histologically, the 5,427  $\mu\text{g/L}$  exposed corals had significantly less mucus in the epidermis and upper gastrodermis compared to the other treatments ( $p<<0.01$ ) after 48 h of exposure (Figure 5A and Figure 5B). While not significantly different, epidermal mucus area in the 640  $\mu\text{g/L}$  corals compared to both control treatments was 19% higher after 48 h of exposure (Figure 5A), indicative of an elevated mucus secretion response in the 640  $\mu\text{g/L}$  corals which has exceeded short-term mucus production capacity in the 5,427  $\mu\text{g/L}$  corals.

Areas of localized epidermal necrosis observed in the 5,427  $\mu\text{g/L}$  corals may follow this apparent exhaustion of mucus production capacity, therefore resulting in significantly increased granular amoebocyte area in the epidermis. Significant treatment effects were found for epidermal amoebocyte area ( $F_{(3,8)}=21.664, p=0.0003$ ) after 48 h of exposure. Post-hoc analysis indicated that epidermal granular amoebocyte area in the 640  $\mu\text{g/L}$  corals was significantly greater than both control treatments ( $p<<0.01$ ) (Figure 5C), and that the 5,427  $\mu\text{g/L}$  corals had significantly greater pigmented granular amoebocyte area in the epidermis ( $p<<0.01$ ), and significantly less granular amoebocyte area in the gastrodermis ( $p<0.05$ ) compared to the other treatments (Figure 5C and Figure 5D). No significant differences were found after the post-exposure recovery period ( $p>0.05$ ).

The pigmented granular amoebocytes common to this genus appear to play a central role in the inflammatory and immune response to tissue injury [37]; thus, of the histological parameters assessed, granular amoebocyte area may be a useful sub-lethal endpoint for this species. This parameter was used to determine a supplemental EC50 of 6,695  $\mu\text{g/L}$  (95% CI: 2,973–10,420  $\mu\text{g/L}$ ), which was slightly more conservative than the EC50 determined from coral condition data.

#### *Comparative toxicity*

The graphically determined 48 h LC50 of 12,123 µg/L estimated in the present study indicates that *P. divaricata* may be less sensitive to hydrocarbon exposure than other marine invertebrate species.

Previously published LC50 values [32, 38] for other species to methylated naphthalenes are not common. For temperate marine arthropods, LC50 values for 2-methylnaphthalene with comparable exposure times (48 h) are reported as 5,000 µg/L for *Cancer magister* [39] and 1,400 µg/L for *Palaemonetes pugio* [22]. For temperate marine molluscs, the range is from 1,910 to 8,130 µg/L 2-methylnaphthalene [22]. Data for naphthalene are more abundant, with organismal LC50 (48 h) ranging from 2,350 µg/L for *Palaemonetes pugio* to 68,000 µg/L for *Katylisia opima* [22]. To date, only one other study has examined toxicity of individual hydrocarbons to adult scleractinian corals, determining an LC50 for fluoranthene of 31.4 µg/L (95% CI: 22.4-44.9 µg/L) and 435.1 µg/L (95% CI: 74.2-∞ µg/L) of the upper and under sides, respectively, of *P. divaricata* branches [9]. As LC50 values for other coral reef organisms ranged from 16 µg/L to 67.5 µg/L [9, 40], this indicates that *P. divaricata* may be similarly less sensitive to fluoranthene, although as with much published data, a lack of quantitative chemistry and inconsistency in experimental protocols makes direct comparisons challenging and uncertain.

## CONCLUSIONS

Previous studies have characterized the effect of petroleum hydrocarbons on corals with a wide variety of assessment metrics, which limit cross-study comparability. In the present study, exposure to 1-methylnaphthalene significantly impacted physical condition, photosynthetic efficiency, and histologic parameters in the shallow-water scleractinian coral *P. divaricata*, although the potential for post-exposure recovery was observed.

A significant contribution of the present study is development and application of a standardized toxicity testing protocol for adult scleractinian corals which considers coral response at multiple levels of resolution and is applicable to many coral species and test scenarios. The present study has generated new hydrocarbon toxicity data for shallow-water scleractinian corals, demonstrating

significant lethal and sub-lethal impacts of the hydrocarbon 1-methylnaphthalene to *P. divaricata*.

Further experimentation utilizing this testing protocol with other single hydrocarbons, both in this

species and with additional coral species will contribute to a more complete picture of hydrocarbon toxicity to scleractinian corals.

*Supplemental Data*—The Supplemental Data are available on the Wiley Online Library at DOI:

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*Data availability*—Readers may contact the primary author (drenegar@nova.edu) for access to the

digital data.

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Figure 1. Recirculating continuous flow exposure apparatus. Water is continuously supplied to a 500 mL glass exposure chamber from an individual 2 L dosing vessel by a multi-channel peristaltic pump (flow rate=5 mL/min) by Viton tubing. 1-methylnaphthalene was passively dosed using polydimethylsiloxane (PDMS) O-rings as a reservoir; 35 O-rings were placed in the stirred dosing vessel and 3 O-rings were placed in the individual exposure chambers. Water samples for hydrocarbon analysis were collected from a sampling port on the exposure outflow.

Figure 2. *Porites divaricata*. Coral physical response to 1-methylnaphthalene at 12 and 48 h of exposure. A) Seawater control, B) 640 µg/L treatment, C) 5,427 µg/L treatment, and D) 25,832 µg/L treatment.

Figure 3. *Porites divaricata*. A) Coral condition scores (mean ± SE) during pre-exposure (at end of pre-exposure), exposure (after 48 h), and post exposure (after 1 wk of recovery) periods, and B) effective quantum yield (mean ± SE) during pre-exposure (at end of pre-exposure), exposure (after 48 h), and post exposure (after 1 wk of recovery) periods. Letters above each bar represent statistical differences between treatments at each time point (a-c;  $\alpha=0.05$ ).

Figure 4. *Porites divaricata*. Histological micrographs of coenenchyme after 48 h of exposure. A) MeOH control coral, B) 640 µg/L exposed coral and C) 5,427 µg/L exposed coral. ep=epidermis, gd=gastrodermis, am=granular amoebocyte, mu=mucocyte. Scale bars=50 µm.

Figure 5. *Porites divaricata*. Histological characteristics (mean ± SE) from coenenchyme after 48 h of exposure. A) Epidermis mucus area %, B) gastrodermis mucus area %, C) epidermis pigmented granular amoebocyte area % and D) gastrodermis pigmented granular amoebocyte area %. pigm. gran. amoe. = pigmented granular amoebocyte. Letters above each bar represent statistical differences between treatments (a-c;  $\alpha=0.05$ ).

Table 1. Measured concentrations of 1-methylnaphthalene ( $\mu\text{g/L}$ ) (mean  $\pm$  SD) for each treatment at 0, 24, and 48 h of the exposure period

Treatment	Mean measured concentrations ( $\pm$ SD) of 1-methylnaphthalene ( $\mu\text{g/L}$ )		
	0 h	24 h	48 h
Seawater Control	$5.9 \pm 2.4$	$7.5 \pm 2.7$	$4.4 \pm 3.3$
MeOH Control	$6.5 \pm 2.8$	$7.1 \pm 2.9$	$3.8 \pm 1.1$
640 $\mu\text{g/L}$	$643.0 \pm 27.7$	$637.3 \pm 37.2$	$639.9 \pm 34.8$
5,427 $\mu\text{g/L}$	$5,021.7 \pm 1,111.4$	$5,575.6 \pm 808.4$	$5,683.7 \pm 783.9$
25, 832 $\mu\text{g/L}$	$26,637.3 \pm 841.8$	$25,762.6 \pm 499.9$	$25,095.0 \pm 1,480.1$

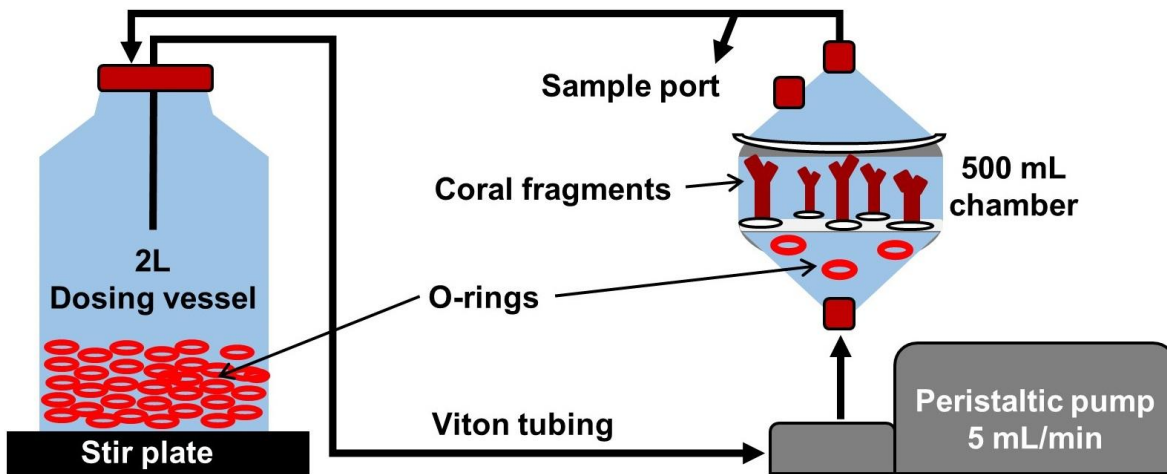


Figure 1

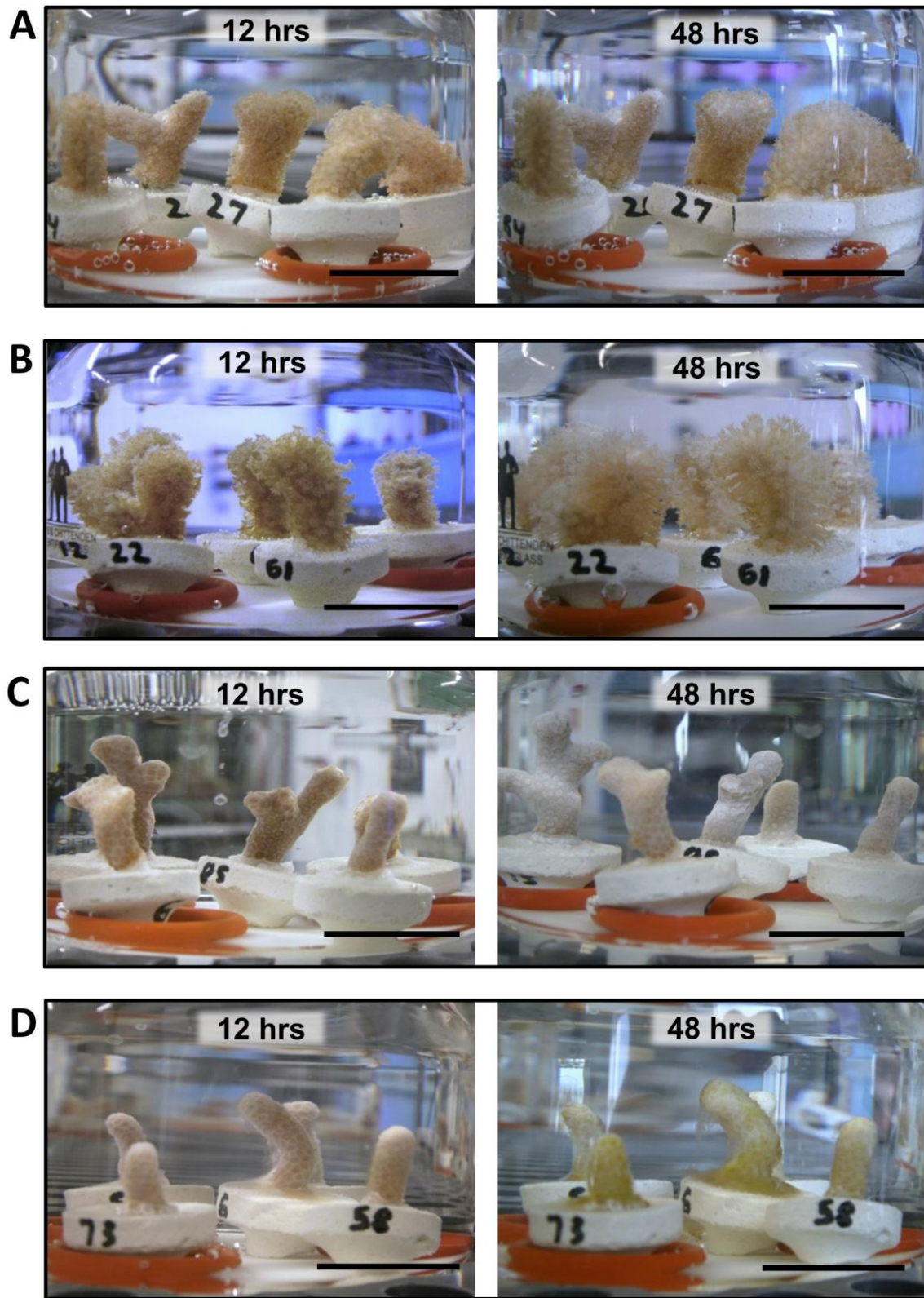


Figure 2

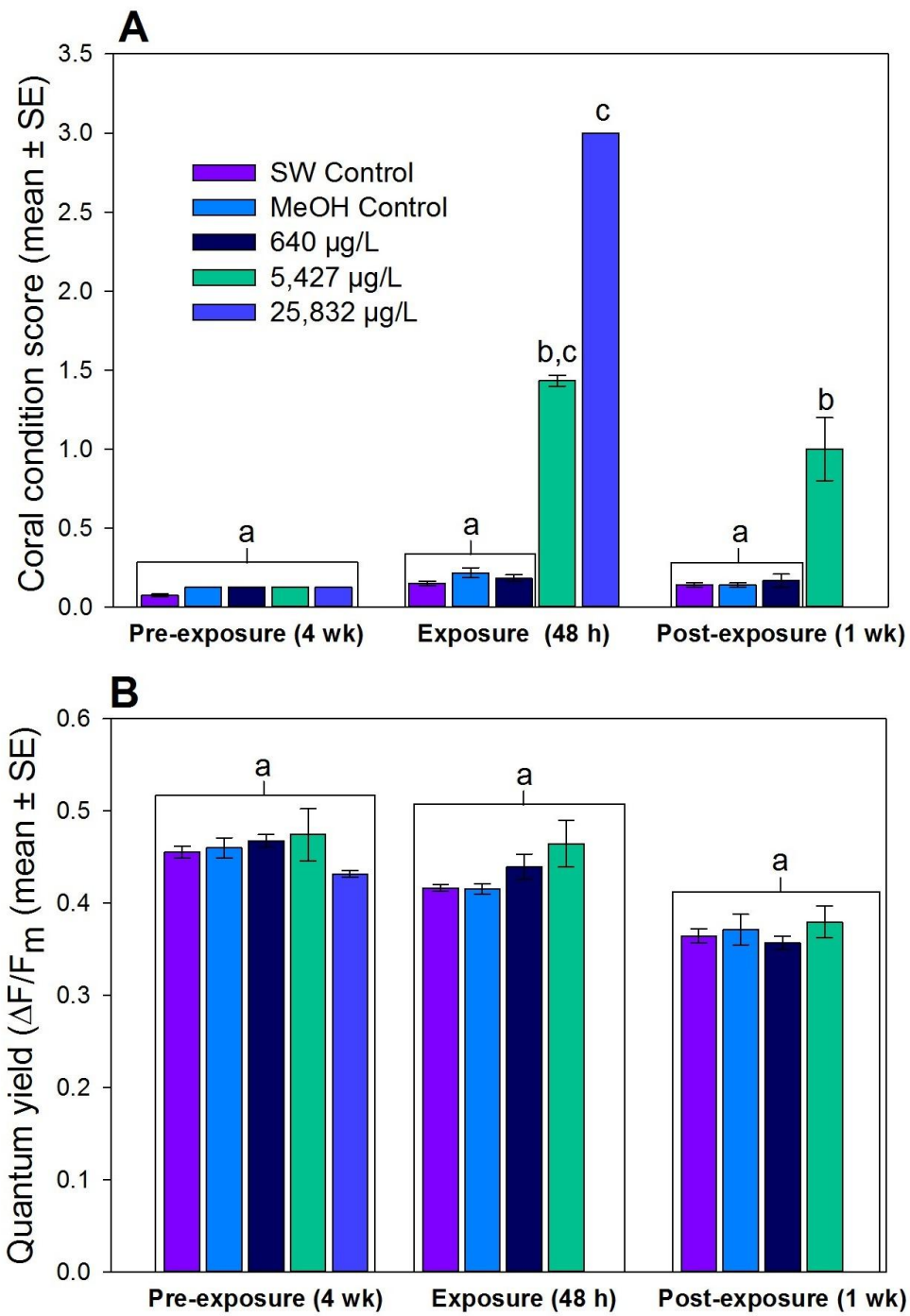


Figure 3

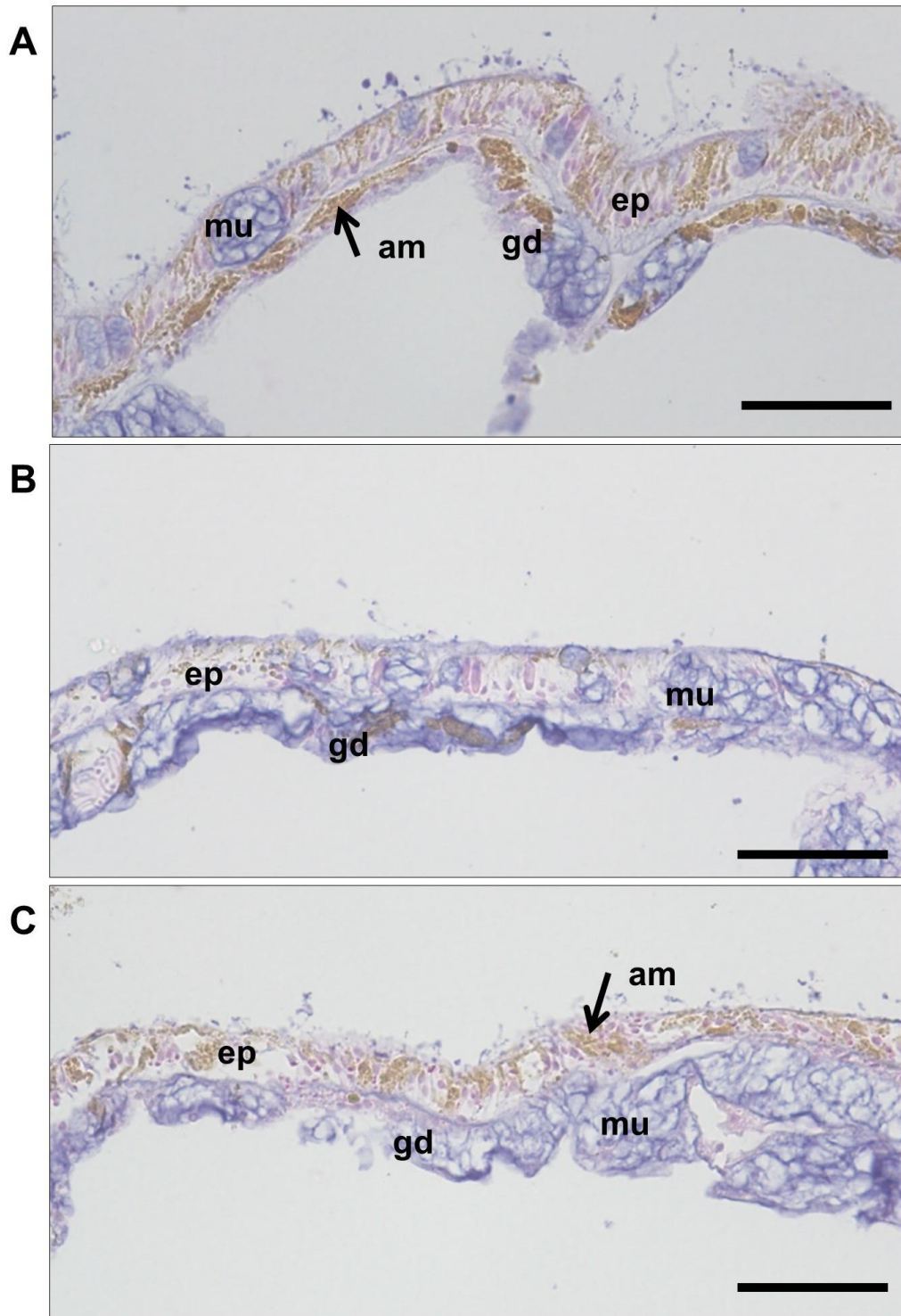
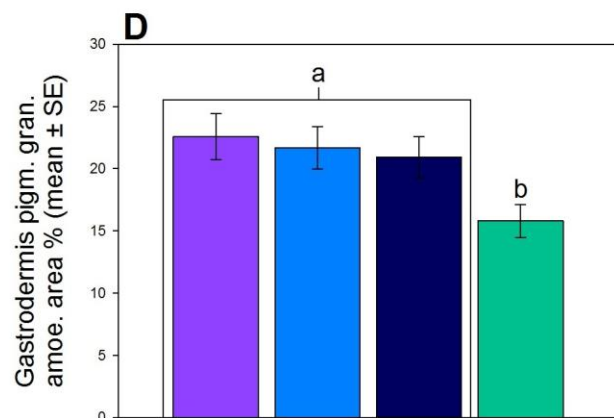
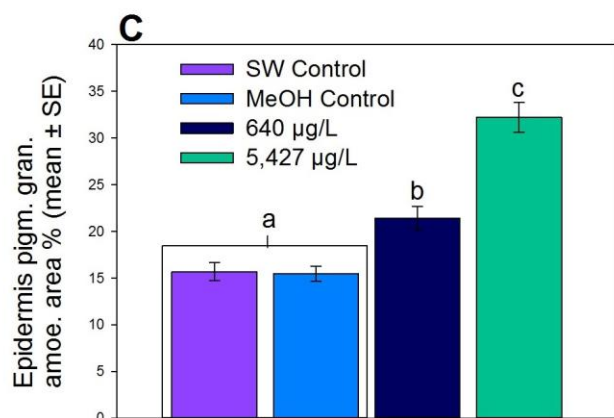
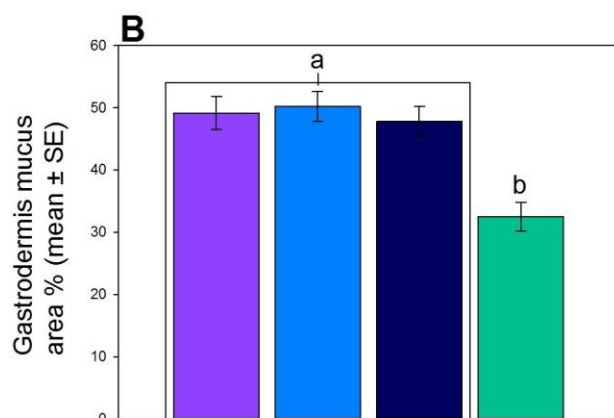
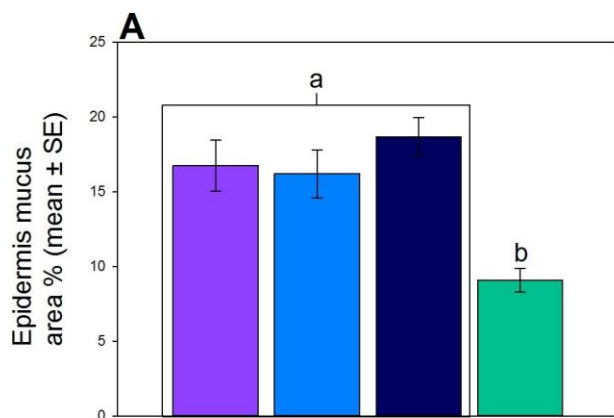


Figure 4



**Figure 5**