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# Effect of Louisiana sweet crude oil on a Pacific coral, *Pocillopora damicornis*

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ABSTRACT

Recent oil spill responses such as the Deepwater Horizon event have underscored the need for crude oil ecotoxicological threshold data for shallow water corals to assist in natural resource damage assessments. We determined the toxicity of a mechanically agitated oil-seawater mixture (high-energy water-accommodated fraction, HEWAF) of a sweet crude oil on a branched stony coral, *Pocillopora damicornis*. We report the results of two experiments: a 96 h static renewal exposure experiment and a "pulse-chase" experiment of three short-term exposure durations followed by a recovery period in artificial seawater. Five endpoints were used to determine ecotoxicological values: 1) algal symbiont chlorophyll fluorescence, 2) a tissue regeneration assay and a visual health metric with three endpoints: 3) tissue integrity, 4) tissue color, and 5) polyp behavior. The sum of 50 entrained polycyclic aromatic hydrocarbons (tPAH50) was used as a proxy for oil exposure. For the 96 h exposure dose response experiment, dark-adapted maximum quantum yield (Fv/Fm) of the dinoflagellate symbionts was least affected by crude oil ($EC_{50}$ = 913 µg/L tPAH50); light-adapted effective quantum yield (EQY) was more sensitive ($EC_{50}$ = 428 µg/L tPAH50). In the health assessment, polyp behavior ($EC_{50}$ = 27 µg/L tPAH50) was more sensitive than tissue integrity ($EC_{50}$ = 806 µg/L tPAH50) or tissue color ($EC_{50}$ = 926 µg/L tPAH50). Tissue regeneration proved to be a particularly sensitive measurement for toxicity effects ($EC_{50}$ = 10 µg/L tPAH50). Short duration (6 – 24 h) exposures using 503 µg/L tPAH50 (average concentration) resulted in negative impacts to *P. damicornis* and its symbionts. Recovery of chlorophyll *a* fluorescence levels for 6 – 24 h oil exposures was observed in a few hours (Fv/Fm) to several days (EQY) following recovery in fresh seawater. The coral health assessments for tissue integrity and tissue color were not affected following short-term oil exposure durations, but the 96 h treatment duration resulted in significant decreases for both. A reduction in polyp behavior (extension) was observed for all treatment durations, with recovery observed for the short-term (6 – 24 h) exposures within 1–2 days following placement in fresh seawater. Wounded and intact fragments exposed to oil treatments were particularly sensitive, with significant delays observed in tissue regeneration. Estimating ecotoxicological values for *P. damicornis* exposed to crude oil HEWAFs provides a basis for natural resource damage assessments for oil spills in reef ecosystems. These data, when combined with ecotoxicological values for other coral reef species, will contribute to the development of species sensitivity models.

*Abbreviations:* ASW, artificial seawater; C.I., confidence interval; DWH, Deepwater Horizon; EQY, effective quantum yield; HEWAF, high-energy water-accommodated fraction; Fv/Fm, maximum quantum yield; ppt, parts per thousand; PAM, pulse amplitude modulated; PSII, photosystem II; PAR, photosynthetically active radiation; PAH, polycyclic aromatic hydrocarbons; TBO, toluidine blue O; tPAH50, sum of 50 polycyclic aromatic hydrocarbons

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## 1. Introduction

Scleractinian coral species are exposed to oil from both chronic input into shallow marine waters and from acute exposures (e.g., spills) (National Research Council, 2003). Annually, approximately 390 million gallons of petroleum products enter the world's oceans, with 54 % coming from human-derived activities such as extraction, transportation and consumption of oil products (National Research Council, 2003). Recent studies indicate that chronic hydrocarbon exposure (e.g., natural seeps, land-based runoff) may impact both the coral animal and its dinoflagellate symbionts (Downs et al., 2012; Jafarabadi et al., 2018). Highly polluted areas such as the Persian Gulf (Sinaei and Mashinchian, 2014) and Jakarta Bay, Indonesia (Baum et al., 2016) have chronic PAH levels between 10 − 385 μg/L (Σ16 and Σ15 PAHs, respectively). Most reported levels of chronic oil input to coral reef waters have been in the ng/L (parts per trillion) range, however (Cheng et al., 2010; Jafarabadi et al., 2018; Zhou et al., 2000). By contrast, the sudden exposure of corals to large oil spill events such as DWH can have catastrophic short-term effects, as the capacity to acclimatize quickly to the petroleum hydrocarbon load is limited, and cellular metabolic processes can be overwhelmed (Downs et al., 2006).

Evaluations of coral physiological condition in response to xenobiotics have included monitoring the loss of the dinoflagellate symbionts (zooxanthellae) or 'bleaching' (Brown, 2000; Douglas, 2003; Jones, 1997), physiological changes such as polyp retraction (Reimer, 1975; Renegar et al., 2016; Wyers et al., 1986) and estimation of percent tissue loss during disease events or exposure to stressors (Reimer, 1975; Renegar et al., 2016; Thompson et al., 1980). It is estimated that stony corals acquire as much as 90 % of the fixed carbon required for growth from zooxanthellae (Muscatine and Porter, 1977); therefore, it is important to evaluate possible oil effects on the coral symbionts. Measuring algal photosynthetic quantum yield under dark- and light-adapted conditions using a pulse amplitude modulating (PAM) fluorometer provides a method to evaluate chlorophyll *a* fluorescence, providing insight on carbon fixation and photosystem II (PSII) damage (Ralph et al., 2005). Coral condition also has been evaluated by monitoring tissue repair of experimentally induced wounds, as slower regeneration rates are associated with increased exposure to stressors (Fisher et al., 2007; Kramarsky-Winter and Loya, 2000; Meesters and Bak, 1993; Moses and Hallock, 2016; Rodriguez-Villalobos et al., 2016; Traylor-Knowles, 2016). Since coral reefs are often in close proximity to sources of oil such as marinas, drilling rigs, tanker routes and shoreline oil refineries, it is important to understand how petroleum spill exposures impact these critical ecosystems. Oil spills are dependent upon ocean currents and tides, forces that may move the slick away within a few hours, or hold the oil in place for days to weeks. While decades of research have documented negative impacts of crude oil exposure on stony coral species, there has been little evidence linking crude oil effects using hydrocarbon chemistry measurements (Negri et al., 2016; Turner and Renegar, 2017).

*Pocillopora damicornis* (Linnaeus 1758), an Indo-Pacific branching stony coral species, was used to determine oil exposure effects (Hoeksema et al., 2014). It is found in areas with oil-related activities (e.g., shipping lanes, oil rigs) and its relatively fast growth rate makes it particularly suitable for use in tissue regeneration assessments (Rodriguez-Villalobos et al., 2016). Previous studies of petroleum hydrocarbon effects on *P. damicornis* indicate that this species is relatively sensitive to exposure compared to other corals (e.g., *Porites*, *Montipora*, *Fungia*), with documented effects on both the coral animal and the algal symbiont (Johannes et al., 1972; Peachey and Crosby, 1995; Reimer, 1975; Rougee et al., 2006; Te, 1992; Villanueva et al., 2011).

Recently the negative effects of measured petroleum hydrocarbons on coral larvae have been demonstrated (Hartmann et al., 2015; Negri et al., 2016; Overmans et al., 2018). Our study focuses on short-term effects of oil spills in coral reef environments on adult *P. damicornis* fragments. We have coupled analytical chemistry measurements of tPAH50 as a proxy for oil exposure with behavioral and physiological endpoints for coral and associated dinoflagellate symbionts to determine ecotoxicological concentration values of acute oil exposures using a 96 h static renewal dose-response experiment. The effect of shorter-term (6 − 24 h) oil exposure scenarios was tested by varying exposure duration of coral to a single high-energy water-accommodated fraction (HEWAF) oil load (62.5 mg/L) over three separate short exposure durations (6, 12, and 24 h) and including an additional 96 h exposure for comparison. In addition, recovery responses in fresh artificial seawater were evaluated by following exposed fragment responses for up to 184 h post treatment. We hypothesize that acute crude oil exposures will result in 1) health effects (polyp retraction, bleaching, tissue loss), 2) a reduction in symbiont chlorophyll *a* fluorescence, and 3) reduced wound healing ability of *P. damicornis*. We expect that recovery of the measured health parameters may be delayed once the stressor is removed.

## 2. Materials and methods

### 2.1. Chemicals

Solvents (pesticide-free acetone, isopropanol, and pesticide-free hexane), Liquinox detergent, hydrochloric acid, sodium sulfate, toluidine blue O and dimethyl sulfoxide were purchased from Thermo Fisher Scientific (Waltham, MA). Dichloromethane and GF/F paper were acquired from VWR International (Radnor, PA). Louisiana sweet crude oil (Mississippi Canyon Block 252) was a gift from the NOAA Office of Response and Restoration and was stored at 4 °C until use. Internal polycyclic aromatic hydrocarbon (PAH) standards used in the quantitative analysis of PAHs were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA).

### 2.2. Equipment preparation

Precleaned glassware was used for all coral exposures and HEWAF preparation and storage. Straight-sided, wide mouth glass jars (500 mL) were used for coral acclimation and dosing (VWR, Radnor, PA, part #89093-982, cleaning protocol A, level PC). Amber glass bottles for the analysis of semivolatiles and pesticides (group 2) were used for HEWAF storage and all dilutions (I-Chem Certified, Chase Scientific Glass, Rockwood, TN). All vessels were rinsed with Tropic Marin artificial seawater (ASW, Tropic Marin Sea Salts, Wartenburg, Germany) prior to use. Glassware, Teflon and metal utensils not previously exposed to crude oil were cleaned thoroughly according to Chapman et al. (1995). Oiled glassware and Teflon-ware reused in generating HEWAFs (graduated cylinders, funnels, etc.) were cleaned according to the method of Forth et al., 2017.

### 2.3. Coral culture

*Pocillopora damicornis*, colonies were purchased from Pacific East Aquaculture (Mardela Springs, MD) and held under the South Carolina Department of Natural Resources non-indigenous species permit #NI17-0401 at the NOAA Coral Culture Facility in Charleston, SC. Eight weeks prior to experiment initiation, colonies were fragmented to generate approximately 120 small (~2.0 cm height) nubbins. Coral nubbins for the tissue regeneration experiment were 3.0 cm, so that when cut, they would meet the target (2.0 cm) height. Each nubbin was attached with cyanoacrylate gel (e.g., superglue gel) to a custom-made Teflon mounting peg (Supplement A). Mounted nubbins were cultured in a glass and Teflon aquarium system (26.0 +/- 0.5 °C) containing ASW (36 ppt) under a 10 h:14 h light:dark cycle. Lighting was provided by two 4 × 39 W T-5 HO light fixtures, each with two AquaSun (UV Lighting Co., Avon, OH) and two Blue Plus (ATI, Denver, CO) 54 W bulbs. The photosynthetically active radiation (PAR) at coral depth ranged from 68 to 77 $\mu mol\, m^{-2}\ s^{-1}$. Spectral analysis (JAZ

spectrometer, Ocean Optics, Largo, FL) of the lighting system showed three major peaks at 430–440, 540–550 and 610 − 620 nm. Corals were fed a mixture of Bio-Pure® frozen brine shrimp nauplii, rotifers, and cyclopods (Hakari, Hayward, CA), Grow Fry Starter (New Life Spectrum®, Homestead, FL) and Reef-Roids (Polyp Lab, Lenexa, KS) three times per week until oil exposure initiation. Accumulated algal biofilms were manually debrided from the Teflon pegs 2–3 days before starting the experimental acclimation.

### 2.4. Coral acclimation

*Pocillopora damicornis* fragments were acclimated in an environmentally controlled room (26 °C) for 72 h prior to starting the experiment. Coral nubbins on Teflon pegs were placed in custom-made Teflon jar stands (Supplement A) and Teflon stands with coral were transferred to clean 500 mL jars. Each was filled with 400 mL ASW (36.0 +/- 0.5 ppt, 26 °C) during the acclimation period to accommodate the 3.0 cm tissue regeneration nubbins. A Teflon air line (attached to house air line and vinyl tubing via a quick-release valve) was placed in each jar and the bubble rate was adjusted (~1–2/s). Lighting and seawater temperature were as described for coral culture conditions. Water changes (100 %) were performed every 12 h. Salinity and temperature on fresh and spent treatment solutions were monitored at each water change.

### 2.5. Crude oil high-energy water-accommodated fraction

The HEWAF was generated using Louisiana sweet crude oil (Macondo source oil collected during the Mississippi Canyon 252 oil spill response) using a Waring™ CB commercial blender according to the methods of Forth et al., 2017. This method generates an oil suspension containing both dissolved hydrocarbon fractions and small oil droplets (Redman, 2015). Briefly, 3.75 L of ASW (36 ppt, 26.0 °C) and 3.75 g of crude oil (1 g/L) were added to the blender under reduced (red) lighting and agitated on low speed for 30 s. The oil-seawater suspension immediately was transferred to a 2.0 L separatory funnel and allowed to separate for 1 h (26 °C). The bottom layer of the unfiltered HEWAF from each vessel (1.5 L total volume) was collected into a clean, rinsed (ASW) 4 L amber glass bottle and used immediately to make the required dilutions (two-fold series, 3.9–250.0 mg/L, nominal oil concentrations). The sum of 50 individual polycyclic aromatic hydrocarbons and alkylated homolog groups (tPAH50) was used as a proxy for crude oil exposure. The tPAH50 results are presented graphically as the geometric mean of fresh and 12 h spent treatment concentrations (see section 2.11 Analytical Chemistry). We also present effect concentrations calculated from chemistry analysis of the fresh (time 0) treatments.

### 2.6. HEWAF 96 h exposure

Corals were exposed to HEWAF dilutions (3.9 − 250 mg/L oil load) in a 96-h static renewal experiment. Treatments were changed every 12 h to keep salinity in check. This 12 h cycle also mimics tidal ebb and flow in the marine environment, which could act to move spilled oil back and forth over a reef. Following the acclimation period and at the onset of the dark period, Teflon stands with coral were transferred with clean Teflon-coated metal tongs to dosing jars containing 350 mL of freshly prepared treatment solution (n = 4). Jars remained open for the duration of the experiment. To reduce placement effects, jars were organized randomly under light fixtures in the temperature-controlled dosing room. One jar with no coral at the highest nominal HEWAF concentration (250 mg/L) was included in the dosing experiment to monitor water quality. Temperature and salinity were measured every 12 h on fresh and spent treatment solutions. Daily pH was measured on spent treatment solutions using a probe connected to a Thermo Orion 5-Star multimeter. Samples (400 µL) for ammonia quantification (sodium salicylate method) were removed from each vessel once daily (12 h spent treatment) and stored at -20 °C until further analysis (Bower and Holm-Hansen, 1980). Water changes (100 %) occurred every 12 h, with newly made HEWAF in clean dosing vessels. Water samples were collected for chemistry analysis at experiment initiation (T0, fresh ASW and fresh HEWAF dilutions). Spent treatment solutions from pooled replicates were also analyzed for tPAH50 at 12 h and 96 h.

### 2.7. Pulse-chase experiment

A pulse-chase experiment was designed to determine effects of acute short-term exposures of coral to oil and to evaluate coral recovery following varying exposure durations. A single HEWAF oil load (62.5 mg/L nominal oil concentration) was selected for this experiment based on effects observed during the 96 h dose-response experiment and reported levels of PAHs documented following oil spills (DIVER, 2017). The five treatment regimens (n = 4) included: an ASW control, 6 h, 12 h, 24 h and 96 h HEWAF exposures (pulse) followed by a recovery period (chase) of 7–11 days in ASW. Coral nubbins were acclimated as above (single replicate for each endpoint per dosing jar), then exposed to the single oil load across treatment times. Coral fragments were transferred to fresh treatment solutions in clean jars every 12 h. Fragments in the 6 h and 12 h treatments received a single pulse of oil treatment, while the 24 h and 96 h treatments were subjected to repeated pulses (2 and 8, respectively). Samples for chemical analysis were collected: fresh control ASW (T0 and 84 h, n = 2) fresh HEWAF (T0, 12 h, 24 h and 84 h, n = 8), spent ASW (6 h, 12 h, 24 h and 96 h, n = 4) and spent HEWAF (6 h, 12 h, 24 h and 96 h, n = 4). Each treatment jar contained two intact 2.0 cm fragments (for health score and photosynthetic quantum yield endpoints) and two 3.0 cm nubbins for tissue regeneration. One tissue regeneration fragment per treatment replicate was used to determine oil effects on wounded coral fragments (cut prior to oil exposure). A second tissue regeneration fragment was exposed to oil intact, cut at the beginning of the recovery period and evaluated when fragments reached the approximate percent tissue regeneration of control fragments. A comparison between control and exposed fragments was used to estimate approximate recovery times following oil exposure. Temperature and salinity were measured on fresh and spent treatment solutions every 12 h, and pH and ammonia were measured every 24 h (during light cycle).

### 2.8. PAM fluorometry

One coral nubbin per treatment replicate was used to determine dark-adapted maximum quantum yield (Fv/Fm) and light-adapted effective quantum yield (EQY) of dinoflagellate symbionts using an Imaging PAM M-series chlorophyll fluorometer (MAXI version, Walz GmbH, Effeltrich, Germany). Each coral fragment was analyzed 2 h before the end of the dark cycle (Fv/Fm) and 2 h before lights went off (EQY). Experimental time points for the 96 h dose-response experiment were −14 h, 34 h and 82 h (dark adaption) and -4, 44 and 92 h (light adaption). Experimental time points for the pulse-chase experiment were -14, 10, 34, 58, 82, 106, 130, 154 and 250 h (dark adaption) and -4, 20, 44, 68, 92, 116, 140, 164 and 260 h (light adaption).

Replicate fragments were evaluated as previously reported (Ralph et al., 2005) (Supplement A). Data were collected on three areas of interest placed on a flat surface in the center of a given fragment and averages for each replicate fragment were calculated.

### 2.9. Health assessment

One 2.0 cm coral fragment in each dosing jar was visually scored daily (middle of light period) for changes in any of the three parameters associated with health condition (Table 1). The scoring criteria were refined to distinguish gradations of change in tissue integrity, tissue color and polyp behavior (modified from De Leo et al., 2016). Care was

**Table 1**
Coral health score rubric. Maximum cumulative score for a healthy coral is 15: five points for color, five points for tissue integrity and five points for polyp extension.

| Score | Polyp Behavior | Color | Tissue loss (%) |
|---|---|---|---|
| 5 | All fully extended | Normal, 100 % color | 0 |
| 4 | Fully extended, few withdrawn | 25 % color loss | 1–25 |
| 3 | Polyps extended 50 % | Pale, 50 % color loss | 26-50 |
| 2 | Polyps extended 10 % | 75 % color loss | 51-75 |
| 1 | No polyps extended | Bleached, with pale areas | 76-99 |
| 0 | Polyp bailout | Totally bleached | 100 |

taken to avoid disturbing the coral during the assessment (e.g., bumping jars), so that maximum polyp extension could be determined.

### 2.10. Tissue regeneration assay

Immediately prior to experiment initiation, 3.0 cm fragments were cropped using clean, stainless steel bone cutters and leaving a flat apical surface. Corals exposed to oil intact during the pulse-chase experiment were cropped immediately following oil exposure (along with control fragments). Each cut fragment (~2.0 cm) was placed into a dosing jar filled with ASW and a bright field image was taken of the cut surface next to a Teflon centimeter rule using a MVX10 research macro zoom microscope with a 0.63x objective (Olympus, Melville, NY) and equipped with a DP71 digital camera (Olympus, Center Valley, PA). Since new tissue growth is translucent (lacks symbionts), coral tissue was stained prior to imaging at experiment termination. Coral fragments were removed from the treatment solution, rinsed in ASW and placed in a jar filled with ASW approximately 1 cm below the cut surface of the coral fragment. The translucent new tissue growth was dyed with a vital stain prior to imaging. Toluidine blue O (TBO, 1 % in DMSO) was diluted to 0.1 % in ASW. Approximately 30 µL of the dilute TBO was placed onto the cut surface of the coral and incubated at room temperature for 3 min. Stain was reapplied as needed (i.e., if it drained away from the coral surface). Fragments were rinsed in ASW and imaged as previously described.

Image analysis was performed using Adobe Photoshop CC 2017. Pixel units were calibrated to the centimeter ruler in each image. Total area of bare skeleton was recorded for each fragment at time 0 and experiment termination. Percent tissue regeneration was determined from the difference in skeletal area between the two values.

### 2.11. Analytical chemistry

The sum of 50 polycyclic aromatic hydrocarbon compounds were determined for fresh and spent treatment solutions (as detailed above for each experiment) and used as a proxy for crude oil exposure (Supplement B). Water samples (130 − 1000 mL) were collected in solvent rinsed, pre-acidified (0.2–1.5 mL 18 % HCl), amber bottles and stored at 4 °C until analysis. Samples were spiked with internal standards (18 deuterated polycyclic aromatic hydrocarbons) and extracted using a liquid/liquid extraction adapted from methods detailed in Reddy and Quinn (1999). Samples were passed through silica solid phase extraction (SPE) cartridges and eluted from the cartridge with hexane and dichloromethane. A recovery standard, $d_{14}$-p-terphenyl, was added prior to GC/MS analysis to measure internal standard recovery. Samples were analyzed on an Agilent 6890/5973 N GC/MS with split/splitless injector and a DB17ms 60 m x 0.25 mm x 0.25 µm analytical column. Calibration for all analytes had a coefficient of determination ($r^2$) greater than or equal to 0.995. Data analyses were performed using MSD Chemstation software. A procedural blank and a PAH reagent spike sample were included in all sample extraction batches (n = 8).

### 2.12. Statistical analyses

Fresh (time 0) and the geometric mean of fresh and 12 h spent tPAH50 measurements were used for statistical analyses of each endpoint. All analyses were performed using SAS v9.4 (SAS Institute Inc., Cary, NC, USA). The response variables for the dose-response experiment (health metric scores, percent tissue regeneration, and PAM fluorometry measurements) met the assumptions for parametric statistics. The model residuals followed the normal distribution and the residual variances were homogeneous. A single factor ANOVA (PROC GLM) was performed on each variable using the ASW treatment as the experimental control. A Dunnett's test for multiple comparisons versus control was performed post-hoc to determine significant differences between treatment groups and control. Alpha was set to 0.05 for all statistical tests. To determine the effective concentration to cause a 50 % effect ($EC_{50}$) in the aforementioned response variables, a three-parameter normal probability (fitted probit) equation was constructed (PROC NLIN METHOD = GAUSS) using the PROBNORM function.

For the pulse-chase experiment, tissue regeneration and PAM fluorometry measurements were analyzed using a two-factor ANOVA with a test for interaction. The two factors were pulse duration (6 h, 12 h, 24 h, and 96 h) and treatment (control or dosed). The model residuals followed the normal distribution and the residual variances were homogeneous. Finding no significant interaction, a test for simple effects was performed at each level of pulse duration to examine if control treatments were significantly different from dosed treatments. Alpha was set to 0.05 for all statistical tests. The health score data from the pulse-chase experiment were analyzed using the non-parametric Kruskall-Wallis test followed by non-parametric multiple comparison tests for treatments versus a control (Zar, 1999).

## 3. Results

### 3.1. Water quality

Temperature and salinity levels remained within normal growth parameters for the course of each experiment (26.0 +/- 0.5 °C, 36 +/- 1 ppt). Total ammonia nitrogen (12 h spent treatment sample) ranged from 0.00-0.05 for the initial dose response experiment and 0.00-0.16 mg/L for the pulse-chase experiment. The pH values for the 96 h exposure were between 8.12 and 8.42 and for the pulse-chase experiment ranged from 8.17-8.59.

### 3.2. Analytical chemistry

Crude oil high-energy WAF mixtures consisted of both dissolved PAHs and droplet oil. Chemical analysis of tPAH50 concentrations in HEWAFs followed expected trends, based on degree of stock dilution. Analysis of Louisiana sweet crude oil HEWAF at time zero (T0, 250 mg/L nominal oil load) resulted in 3971 µg/L tPAH50 with roughly 50 % reduction in each subsequently lower treatment dilution (Supplement B.1). After 12 h in the dosing jar, HEWAF tPAH50 concentrations were reduced by at least 88 % in all treatments. For example, the 7.8 mg/L oil loading treatment tPAH50 was measured in the fresh treatment solution as 62 µg/L and decreased to 8 µg/L tPAH50 after 12 h under experimental conditions (Supplement B.2).

Chemical analysis of the pulse-chase experimental treatment also exhibited ~86 % reduction of tPAH50 concentration over 12 h. Initial (fresh, load 62.5 mg/L) average tPAH50 concentrations in eight HEWAF preparations was 503 µg/L (Supplement B.3; percent relative standard deviation ~8 %). The 6 h spent treatment solution retained 131 µg/L tPAH50. Repeated spent treatment analysis after 12 h resulted in average tPAH50 concentrations ranging from 64 to 79 µg/L (Supplement B.3). Spent media tPAH50 concentration was reduced by approximately 74 % after 6 h vs 86 % after 12 h. Individual PAH50

concentrations from the pulse-chase experiment are detailed in Supplement B.4.

### 3.3. Effect of 96 h HEWAF concentrations on coral and symbionts

#### 3.3.1. HEWAF impacts to symbiont chlorophyll a fluorescence

Following a static 12 h renewal exposure of *P. damicornis* fragments to dilutions of HEWAF (12 – 945 µg/L tPAH50, geometric mean of fresh and 12 h spent treatment), oil-exposed symbiont Fv/Fm and EQY were decreased significantly compared to controls ($p < 0.05$, Fig. 1). A reduction in dark-adapted chlorophyll *a* fluorescence was observed with an 82 h exposure (Panel A). Decreased fluorescence was noted for EQY vs Fv/Fm (Panel B). Table 2 shows the effect concentrations for the tPAH50 exposures. At 82 h the oil exposure resulted in an Fv/Fm $EC_{50}$ value of 913 µg/L tPAH50 (95 % C.I. = 862–968), reported as the geometric mean of T0 and 12 h spent treatment. The $EC_{50}$ value for EQY at 92 h was 428 µg/L tPAH50 (95 % C.I. = 375–488). The $EC_{50}$ values calculated using the time zero (fresh) treatment chemistry data were 3804 and 1640 µg/L tPAH50 for Fv/Fm and EQY, respectively (Table 2). Response curves for Fv/Fm and EQY are shown in Supplement C.1.

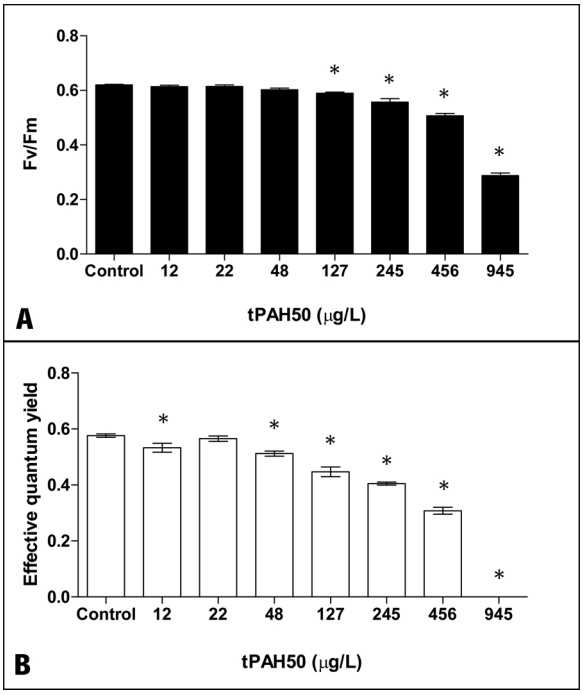


**Fig. 1.** *Pocillipora damicornis* symbiont chlorophyll *a* fluorescence measurements following crude oil exposure. Panel A: dark-adapted maximum quantum yield after 82 h; Panel B: light-adapted effective quantum yield after 92 h. Total PAH50 (tPAH50) is reported as the sum of 50 polycyclic aromatic hydrocarbons. Maximum quantum yield (Fv/Fm, panel A) was reduced significantly (indicated by *) for treatments of 127 µg/L tPAH50 and higher after 82 h of exposure ($p < 0.05$). Effective quantum yield (EQY, panel B) was significantly lower ($p < 0.05$) following exposure with 12 µg/L tPAH50 for 92 h. Effective quantum yield was zero for the 945 µg/L tPAH50 treatment. Total PAH50 values are reported as the geometric mean of fresh and spent treatment solution (refreshed every 12 h).

#### 3.3.2. General coral health effects of HEWAF concentration exposure

*Pocillopora damicornis* fragments exposed to increasing concentrations of HEWAF resulted in significantly decreased ($p < 0.001$) health rubric scores at 89 h (Fig. 2, Supplement D). Tissue loss was first observed for three fragments at 65 h (245 and 456 µg/L tPAH50, geometric mean). Tissue loss continued for the three highest treatments until experiment termination. Tissue loss was most severe for the 945 µg/L tPAH50 treatment, with three of four fragments losing 50–75 % tissue by 89 h (Fig. 2, Panel A). Coral fragments started bleaching by 31 h for the 22 – 945 µg/L tPAH50 treatments, but at 89 h was significant only for the 945 µg/L tPAH50 treatment (Fig. 2, Panel B). A decline in health was driven by polyp behavior (reduced 3–4 points within the first 17 h of exposure), with treatments of 127 µg/L tPAH50 and higher significantly different from the control at 89 h (Fig. 2, Panel C). Some polyp recovery was noted in the 12 µg/L tPAH50 treatment at 41 h post treatment initiation. The $EC_{50}$ values for tissue integrity were 806 µg/L tPAH50 (geometric mean) and 3296 µg/L tPAH50 (fresh treatment), while tissue color resulted in $EC_{50}$ values of 926 (geometric mean) and 3280 (fresh treatment) µg/L tPAH50, respectively (Table 2). Polyp behavior was the most sensitive endpoint in the visual health assessment, with $EC_{50}$ values ranging from 27 (geometric mean) to 84 (fresh) µg/L tPAH50 (Table 2). Dose-response curves with 95 % confidence intervals for each health metric are presented in Supplement C.2.

#### 3.3.3. Inhibition of coral tissue regeneration by crude oil HEWAF concentrations

The tissue regeneration assay was most sensitive to crude oil HEWAF exposure. Tissue regeneration was inhibited in the 96 h dose response experiment at all tested concentrations (Fig. 3, Panel A, $p < 0.005$). Fig. 4 shows representative coral fragments at time zero (after initial cut) and at 96 h (with TBO tissue staining). Treatments of 12 – 127 µg/L tPAH50 (geometric mean of fresh and 12 h spent treatments) had little to no tissue regeneration compared to the control. Increasing tissue loss was observed for fragments subjected to 245 – 945 µg/L tPAH50. The tissue regeneration $EC_{10}$ values ranged from 3 (geometric mean) to 7 (fresh) µg/L tPAH50 and $EC_{50}$ values were 10 (geometric mean) and 29 (fresh) µg/L tPAH50 (Table 2). The response curve for tissue regeneration is shown in Supplement C.3.

**Table 2**
Reported effect concentrations for the 96 h HEWAF dose-response exposure using fresh (time 0) treatments or the geometric mean (Geomean) of fresh and 12 h spent treatment solutions (units = µg/L tPAH50). C. I. = 95 % confidence interval, Fv/Fm = maximum quantum yield, EQY = effective quantum yield, Tissue reg. = tissue regeneration, Tissue integ. = tissue integrity, Polyp retract. = polyp retraction.

| Endpoint | Fresh $EC_{10}$ (C. I.) | Fresh $EC_{50}$ (C. I.) | Geomean $EC_{10}$ (C. I.) | Geomean $EC_{50}$ (C. I.) |
|---|---|---|---|---|
| Fv/Fm (82 h) | **1193** (1051-1355) | **3804** (3575-4049) | **330** (292-372) | **913** (862-968) |
| EQY (92 h) | **632** (472-847) | **1640** (1429-1882) | **176** (13-234) | **428** (375-488) |
| Tissue reg. (96 h) | **7** (1-52) | **29** (15-57) | **3** (0.4-16) | **10** (6-19) |
| Tissue integ. (89 h) | **711** (431-1174) | **3296** (2595-4187) | **202** (128-319) | **806** (649-1000) |
| Tissue color (89 h) | **111** (15-853) | **3820** (1741-8385) | **36** (5-234) | **926** (450-1907) |
| Polyp retract. (89 h) | **8** (2-23) | **84** (50-139) | **3** (1-9) | **27** (17-43) |

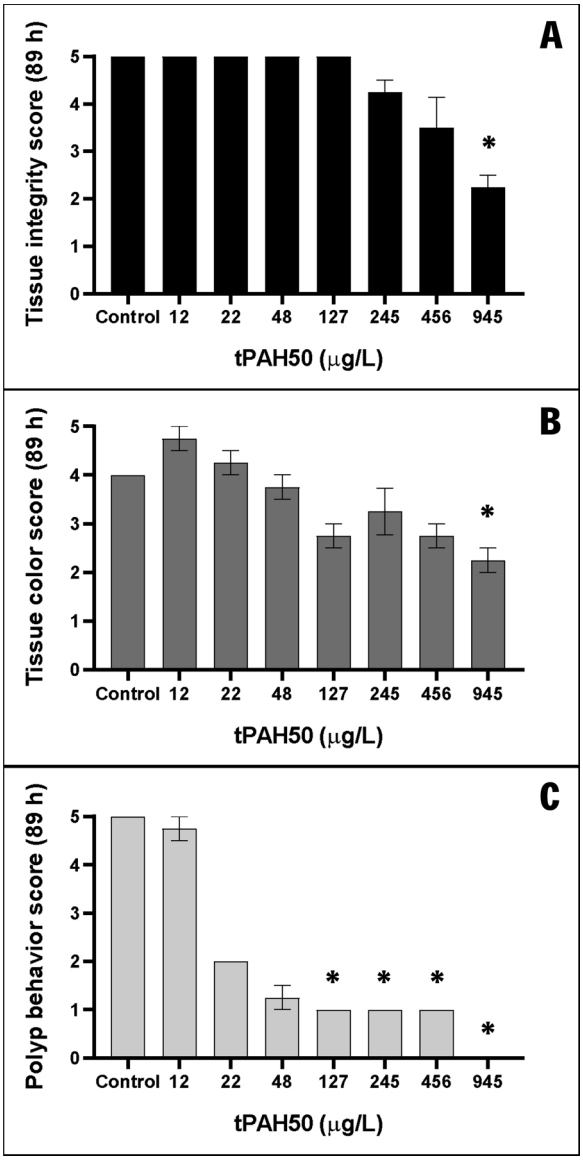


**Fig. 2.** *Pocillopora damicornis* health status effects after 96 h HEWAF exposure. Oil treatments are reported as the sum of 50 polycyclic aromatic hydrocarbons (tPAH50). Panel A: tissue integrity metric, Panel B: tissue color metric and Panel C: polyp behavior metric. Treatments marked with an asterisk (*) were significantly different from the artificial seawater control at $p < 0.001$. Concentrations of tPAH50 are reported as the geometric mean of fresh and spent HEWAF treatment, which was refreshed every 12 h.

### 3.4. Pulse-chase experiment: effects of oil exposure duration

#### 3.4.1. Symbiont chlorophyll a fluorescence effects

Shorter durations of a single HEWAF dose (266 µg/L HEWAF tPAH50 concentration, 6 h geometric mean; 189 µg/L HEWAF tPAH50 concentration, 12 h geometric mean) significantly affected ($p < 0.05$) symbiont chlorophyll *a* fluorescence (Fv/Fm and EQY, Fig. 5). Maximum quantum yield (Fv/Fm) impacts were variable at the 10 h time point with significant reduction for the 12 h and 96 h exposure durations (Fig. 5, Panel A). The 12 h exposure fragments fully recovered within 24 h. The Fv/Fm of the 96 h HEWAF exposure required 34 h to recover to the level of the controls following placement in fresh ASW. The EQY for 6, 12, 24 and 96 h HEWAF exposures was significantly decreased at the 20 h time point (Fig. 5, Panel B). Complete EQY recovery for the 6, 12 and 24 h HEWAF exposures occurred by 92 h (68 – 86 h) after placement in fresh ASW. Recovery time for *P. damicornis* symbionts with significant impacts to EQY after a 96 h HEWAF exposure was between (68 – 164 h post-treatment).

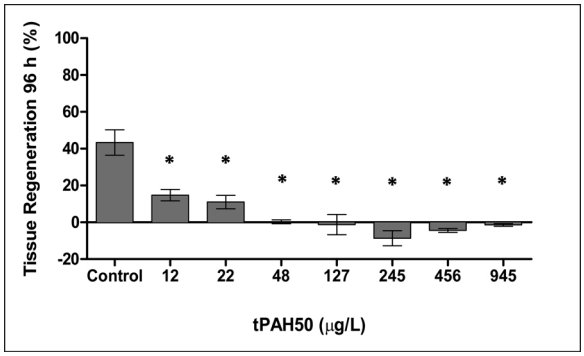


**Fig. 3.** Percent tissue regeneration for *Pocillopora damicornis* following 96 h HEWAF exposure. Oil dose is reported as the sum of 50 polycyclic aromatic hydrocarbons (tPAH50). Treatments designated with an asterisk (*) were significantly different from the control ($p < 0.005$). Total PAH50 values are reported as the geometric mean of fresh and spent treatment solution (refreshed every 12 h).

#### 3.4.2. Coral health score effects

Decreased health effects were observed for *P. damicornis* fragments exposed to crude oil HEWAF. Tissue integrity and tissue color were not affected by the short-term (6 – 24 h) oil exposures; however, immediate polyp retraction was observed in response to all pulse (exposure) durations compared to the no pulse control (Fig. 6). There was little (< 5 %) to no tissue loss for coral fragments in the 6, 12 or 24 h oil treatments and these treatment durations did not result in significant effects when compared to the no treatment control (Fig. 6, Panels A, D). Coral fragments in the 96 h oil treatment exhibited a loss of tissue integrity after 89 h of exposure and this tissue loss increased with time. Coral tissue for all oil treatment durations partially bleached over time; the 96 h treatment duration resulted in significant differences ($p < 0.05$) from the control between 65 and 185 h (Fig. 6, Panels B, E). *Pocillopora damicornis* fragments in the control also showed a very slight loss of color over time, especially after 185 h. Low initial polyp behavior scores at – 7 h (before treatment started) were due to disturbing fragments during photography (Fig. 6, Panel C). Control fragments returned to full extension at 17 h. Polyps exhibited normal behavior (fully extended) for the 6, 12 and 24 h HEWAF following placement in fresh ASW; the normal response was delayed for 24 – 53 h (Fig. 6, Panel C and Supplement E). Fragments exposed to oil for 96 h did not fully extend polyps in the recovery period (up to 233 h). This resulted in a significant difference ($p < 0.05$) for the 96 h oil exposure when compared to the no pulse control after 65 h HEWAF treatment (Fig. 6, Panel F).

#### 3.4.3. Impacts to tissue regeneration

*Pocillopora damicornis* fragments cut prior to exposures and subjected to short-term HEWAF exposures (189 µg/L, 12 h geometric mean; 266 µg/L, 6 h geometric mean tPAH50) showed significantly ($p < 0.0001$) decreased tissue regeneration after 72 h, with tissue loss noted for the longest exposure (Fig. 7, Panel A). Following placement in fresh seawater, delayed tissue regeneration times were observed for all HEWAF treatments compared to the controls at 72 h (approximately 50 % tissue regeneration). The 6 h oil treatment resulted in a 48 h lag in recovery, the 12 h treatment had a 72 h lag and the 24 h treatment resulted in 84 h delayed recovery. The cut coral fragments in the 96 h exposure did not recover to the level of the 72 h controls by the end of the experiment (168 h post treatment). Significant impacts ($p < 0.0001$) to oil-exposed intact coral fragments were also noted (Fig. 7, Panel B). Intact fragments cut following oil exposure and placed

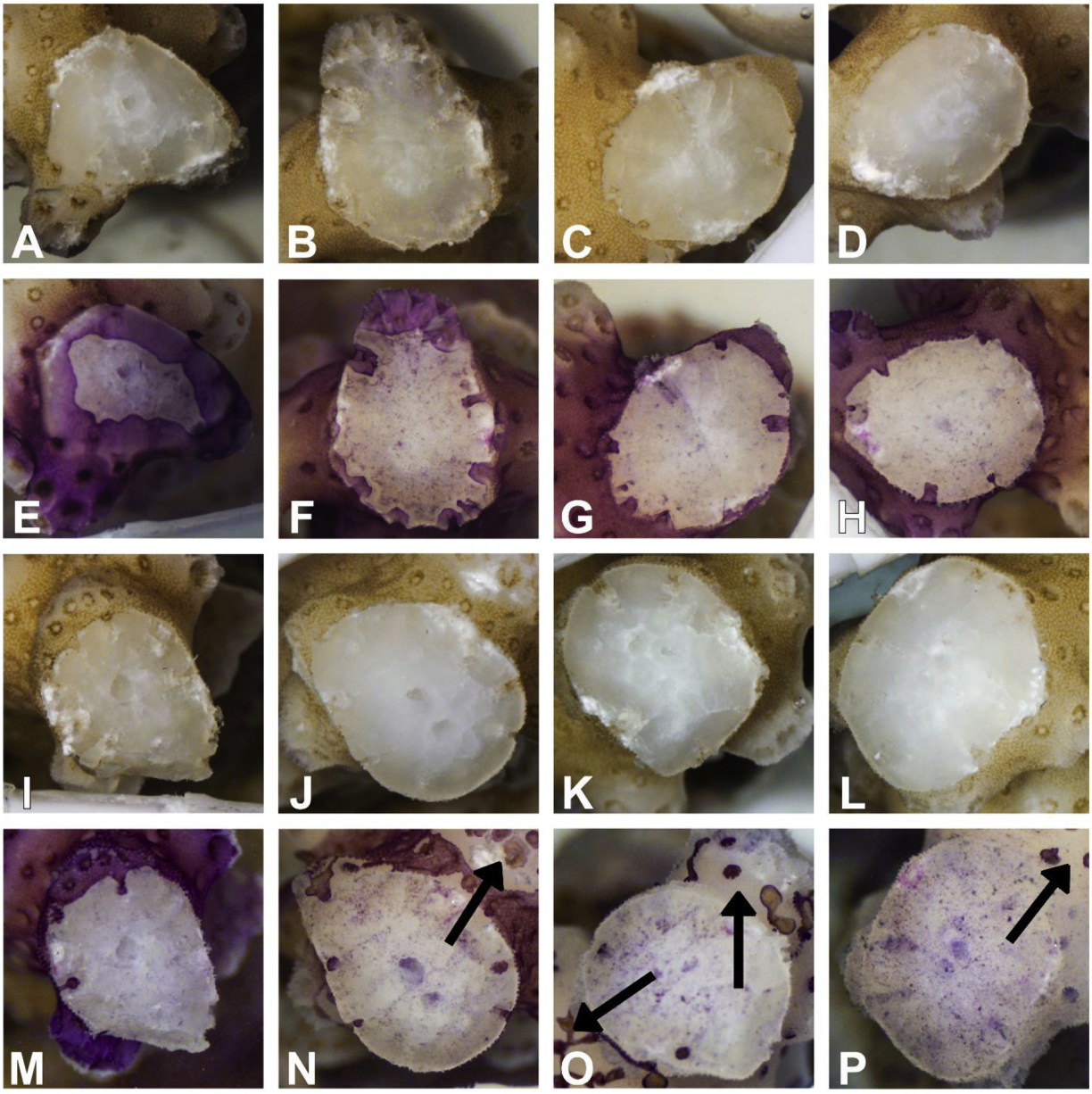


**Fig. 4.** *Pocillopora damicornis* tissue regeneration images. Images are shown at time 0 following initial cut (Panels A-D and I-L) and after 96 h treatment, stained with toluidine blue O (Panels E-H and M-P). Panels A and E = ASW control, panels B and F = 12 µg/L tPAH50, panels C and G = 22 µg/L tPAH50, panels D and H = 48 µg/L tPAH50, panels I and M = 127 µg/L tPAH50, panels J and N = 245 µg/L tPAH50, panels K and O = 456 µg/L tPAH50 and panels L and P = 945 µg/L tPAH50. Areas of tissue loss for treatments at and above 245 µg/L tPAH50 are indicated by arrows in panels N, O and P. Total PAH50 values are reported as the geometric mean of fresh and spent treatment solution (refreshed every 12 h).

in fresh seawater resulted in significantly slowed tissue regeneration 72 – 84 h post treatment compared to controls cut at the same time.

## 4. Discussion

Oil exploration, production and transport often intersect spatially with shallow coral reef ecosystems throughout the tropics and subtropics, presenting numerous threats of exposure through spills, groundings or wellhead blowouts, as in DWH. This is the first study to demonstrate ecotoxicological effects of acute crude oil HEWAF exposures on the adult life stage of the ecologically important shallow water coral species, *P. damicornis,* using the surrogate, tPAH50. Total PAH50 has been used as a proxy for oil toxicity to other marine organisms (Esbaugh et al., 2016; Incardona et al., 2014; Stieglitz et al., 2016), providing a platform to compare toxicity between species and contaminant type that are suitable for oil spill-related ecological risk assessments and natural resource damage assessments. We emphasize that our experiments were performed under laboratory light conditions (no ultraviolet light, UV). As it has been demonstrated that UV light enhances toxicity of hydrocarbon compounds for marine organisms (Alloy et al., 2016; Finch et al., 2017; Sweet et al., 2017), the coral toxicity data we report are likely conservative values.

### 4.1. Ecotoxicological Potential of Louisiana sweet crude oil to coral

#### 4.1.1. Oil effects on symbiont photosynthesis

We observed that symbiont Fv/Fm was impacted by crude oil exposure ($EC_{10}$ = 330 µg/L tPAH50, geometric mean) and this was further reduced to 176 µg/L tPAH50 following *P. damicornis* exposure to light. The reported effect concentration values for *P. damicornis*

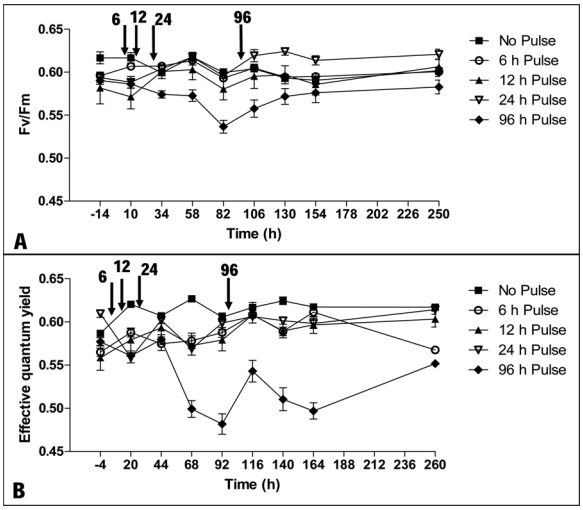


**Fig. 5.** Chlorophyll *a* fluorescence measurements of *Pocillopora damicornis* algal symbionts following short-term crude oil exposure. The HEWAF exposure durations of 6, 12, 24 and 96 h (indicated by arrows) contained 266 µg/L tPAH50 (6 h duration) or 189 µg/L tPAH50 (12, 24 or 96 h duration) (geometric mean of fresh and spent HEWAF treatment). Maximum quantum yield (Fv/Fm, evaluated two hours before the light cycle began) was reduced significantly ($p < 0.05$) for the 12 h and 96 h pulse durations at the 10-h time point (Panel A). No significance was observed for Fv/Fm in the 6 h and 24 h pulse durations. Fragments exposed to a 12 h pulse recovered Fv/Fm within 24 h after treatment ended and fragments exposed for 96 h recovered to the level of the controls within 34 h post treatment (130 h time point). Effective quantum yield (EQY, measured two hours before light cycle ended) for all oil pulse durations was impacted significantly compared to controls at the 20 h time point (Panel B, $p < 0.05$). The EQY for 6, 12 and 24 h pulse exposures recovered to the level of the controls by 92 h. The 96 h pulse duration was significantly different from the control up to 68 h post treatment and recovered by the end of the experiment (260 h).

symbionts were 1–2 orders of magnitude higher than for tissue regeneration, indicating that PAHs are less toxic to the algal symbiont than to the coral animal. Chlorophyll *a* fluorescence measurements are valuable indicators of the state of PSII in plants. Exposure to light naturally damages PSII reactions centers (photoinhibition) and dark adaptation provides time for photosynthetic reaction centers in the chloroplast to repair damage normally resulting from exposure to sunlight. Photoinhibition is exacerbated when light is combined with various other environmental stressors, yielding an increase in damaged PSII reaction centers that cannot be repaired during the dark cycle (Nishiyama et al., 2001; Ralph, 2000; Takahashi and Murata, 2005). Since symbiotic zooxanthellae have been reported to contribute as much as 90 % of the fixed carbon required for growth of scleractinian corals (Muscatine and Porter, 1977), impacts to PSII also have severe impacts on coral nutrition, and in turn on growth and other physiological processes (e.g., reproduction, defense, wound repair, etc.).

Oil exposure has been shown to impact photosynthetic output in several ways. Physical barriers created by the oil can block sunlight required for photosynthesis or decrease gas exchange (Baker, 1970). Reductions in chlorophyll content have been observed in response to oil exposure of corals (Baruah et al., 2014; Jafarabadi et al., 2018; Olaranont et al., 2018). In addition, Jafarabadi et al. (2018) have shown that PAHs can penetrate both coral tissues and zooxanthellae membranes. Since chlorophyll-peridinin complexes of many autotrophic dinoflagellates incorporate both hydrophilic and lipophilic proteins, cellular penetration of PAH compounds could act to rupture the photosynthetic assembly, with pigments subsequently degraded.

Similar to our study, decreases in Fv/Fm from oil exposure have been reported for *Acropora formosa* (Mercurio et al., 2004) and *Porites divaricata* (Guzmán Martínez et al., 2007) symbionts with effective concentrations of hydrocarbon in the ppb (µg/L) range. However *Pocillopora verrucosa* exposure to a diesel fuel WAF (415 µg/L total PAH, geometric mean, *our calculation*) resulted in no significant effect on symbiont Fv/Fm in a static 84-h test (Kegler et al., 2015). Additionally, Renegar et al. (2016) found no effect on Fv/Fm following a 48-h exposure of *P. divaricata* to a single PAH compound, 1-methylnaphthelene in a continuous-flow system (640-25,832 µg/L). While experimental design (static, continuous flow, or static renewal test), exposure duration, lighting and the use of different PAH mixtures may have played a role in the contrasting results, it is possible that variable rates of PSII electron transport among zooxanthellae species are also a factor (Cantin et al., 2009). Additionally, variable optical properties of corals (fluorescent proteins, chromoproteins) can confound PAM-based fluorescence measurements (Mayfield et al., 2014; Wangparseurt et al. (2019)). We observed significant tissue color loss in the visual health score only at the highest HEWAF treatment (945 µg/L tPAH50), but significant decreases in Fv/Fm at much lower concentrations (to 48 µg/L tPAH50, Fig. 1). While it is possible that symbionts could be dead or dying within the coral tissues, it also is possible that reduction in Fv/Fm for oil-exposed corals may be due to PAH effects on the photosynthetic machinery. Further research to understand mechanisms of coral symbiont photosynthetic responses to crude oil exposure is needed.

#### 4.1.2. Oil effects on polyp behavior and tissue integrity

*Pocillopora damicornis* exhibited severe polyp retraction in response to crude oil HEWAF exposure ($EC_{10}$ = 3 µg/L tPAH50, geometric mean). Corals can respond in a limited number of ways to xenobiotic exposure. Mucocytes located in the epidermal cell layer produce a lipopolysaccharide mucus layer, which acts as a buffer to the immediate environment. Mucus production can be stimulated, and it can bind and remove potentially harmful chemicals such as oil (Bak and Elgershuizen, 1976). Corals also retract polyps in response to irritants such as crude oil (Knap, 1987; Lewis, 1971; Reimer, 1975). This action closes off the gastrodermal cavity, possibly delaying or limiting effects of toxic compounds on the gastrodermal cells not protected by a mucus layer. Since coral species such as *P. damicornis* extend polyp tentacles as a normal heterotrophic feeding behavior, sustained polyp retraction can reduce feeding activity, and potentially nutrient intake. When the concentration of a contaminant reaches a threshold that overcomes these natural defense mechanisms, biochemical and cellular processes break down, which can result in tissue degradation.

We also observed that *P. damicornis* lost tissue in response to crude oil HEWAF exposure ($EC_{10}$ = 202 µg/L tPAH50). Adult coral mortality is usually associated with complete tissue loss from the skeleton. While corals can recover from partial tissue losses (Kramarsky-Winter and Loya, 2000), even small tissue lesions can have a negative impact on colony health by increasing chances of disease by opportunistic microorganisms (Page and Willis, 2008). In the wild, tissue integrity changes likely affect feeding activity, increase susceptibility to disease or encourage algal overgrowth, and/or decrease photoprotective mechanisms due to chromoprotein or fluorescent protein loss (Lamb et al., 2014; Lirman, 2001; Salih et al., 2000).

Results from this study and others indicate that corals elicit an innate protective response to oil exposure (Bak and Elgershuizen, 1976; De Leo et al., 2016; Elgershuizen and De Kruijf, 1976; Frometa et al., 2017; Lewis, 1971; Reimer, 1975; Renegar et al., 2016; White et al., 2012; Wyers et al., 1986). We observed significant negative gross health effects for *P. damicornis* in an acute 96 h static renewal test. Renegar et al. (2016) recently reported a health score $EC_{50}$ value of 7442 µg/L tPAH50 for *P. divaricata* exposure using a single PAH analyte, 1-methylnaphthalene. We can make no direct comparison with our study due to differences in health metric reporting, PAH composition and exposure duration, however. *Porites divaricata and P. damicornis* are both 'weedy' coral species, often found in disturbed environments or when reef species complexity is low (Darling et al., 2012; Newman et al., 2015; Smith et al., 2013). Increased relative sensitivity of *P. damicornis*

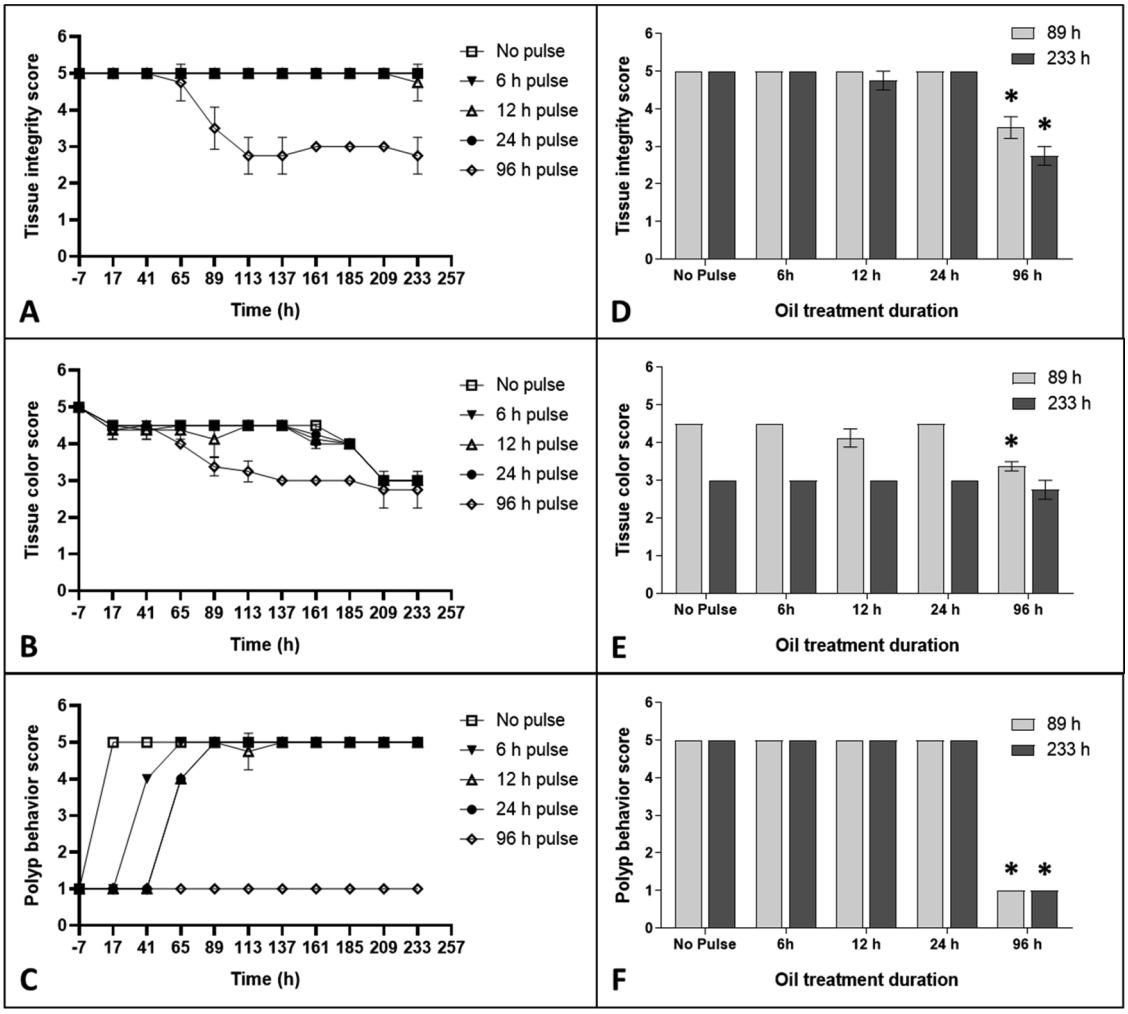


**Fig. 6.** *Pocillopora damicornis* health status scores following short-term HEWAF pulse durations (189-266 µg/L tPAH50, refreshed every 12 h). Coral fragments were evaluated at 2 pm daily. Initial evaluation at -7 h included polyp retraction for all treatments following image analysis. Tissue integrity (Panels A and D) and tissue color (Panels B and E) were not affected by short-term (6-24 h) oil exposures. Coral fragments exposed to HEWAF for 96 h lost tissue and did not recover to the level of the controls after one week in fresh artificial seawater (Panel D, * = significant at $p < 0.05$). Tissue color for the 96 h treatment duration was significantly reduced (indicated by *, $p < 0.05$) from 65 to 185 h (Panel E). All treatments (including controls) had reduced tissue color scores after 185 h. Polyp behavior was significantly affected by all treatment durations (Panels C and F). Recovery for the 6 h (266 µg/L tPAH50), 12 h and 24 h (189 µg/L tPAH50) exposures occurred within 53 h after treatment ceased (Panel C). The 96 h pulse (189 µg/L tPAH50) exposure had significantly reduced (indicated by *, $p < 0.05$) polyp behavior at 89 h and at 137 h post treatment (233 h) time point (Panel F). Total PAH50 is the geometric mean of fresh and spent (6 or 12 h) HEWAF solutions.

compared to other corals has been demonstrated for both natural and anthropogenic stressors, however (Reimer, 1975, Te et al., 1998; Stimson et al., 2002; Downs et al., 2016). Our method of static renewal (vs continuous flow for the *P. divaricata* experiment) also may have contributed to increased toxicity of crude oil to *P. damicornis*. As the 12 h static renewal mimics tidal ebb and flow in shallow marine waters, it suggests that the action of tidal forces to move oil slicks back and forth over a coral reef may increase detrimental effects of oil exposure to stony corals.

#### 4.1.3. Oil effect on tissue regeneration

Tissue regeneration in *P. damicornis* was significantly affected in the 96 h HEWAF exposure at all tested doses (as low as 12 µg/L tPAH50), demonstrating that it is a particularly sensitive quantitative assay for coral toxicology evaluations. Corals are susceptible to wounds from a variety of natural (e.g., fish bites, storms, disease) and anthropogenic (e.g., boat groundings, diver interactions, pollution) causes and regeneration of tissue over bare skeleton requires significant energy input. Wound healing has been used previously as a field assessment tool to gauge coral condition (Dustan et al., 2008; Fisher et al., 2007; Moses and Hallock, 2016). Coral colonies that cannot undergo lesion repair are susceptible to infection, infiltration by boring organisms or algal overgrowth, often leading to poor reproductive output, slowed growth and death (Meesters and Bak, 1993; Rinkevich and Loya, 1979). Kramarsky-Winter and Loya (2000) first demonstrated how tissue repair is dependent upon intrinsic (size, reproductive state) and extrinsic (season, water temperature) factors by comparing field and laboratory wound healing rates. We have shown that this assay is amenable to laboratory toxicology studies with fast-growing, branching corals, which are easily fragmented. Slower-growing corals, such as *Orbicella, Dendrogyra,* or *Diploria* spp., may not be easily wounded, nor exhibit enough tissue regrowth to be utilized in shorter timeframe toxicological studies using this assay, however.

While the precise mechanism of oil toxicity is not well understood, we know that corals can bioaccumulate hydrocarbons (Burns and Knap, 1989; Jafarabadi et al., 2018; Ko et al., 2014; Peters et al., 1981; Sabourin et al., 2013) and uptake of these hydrophobic compounds can result in disruption of cellular biochemical and physiological processes. Exposure to oil is linked with a decrease in lipid biosynthesis (Cook and Knap, 1983) and disruption of cellular protein production (Rougee

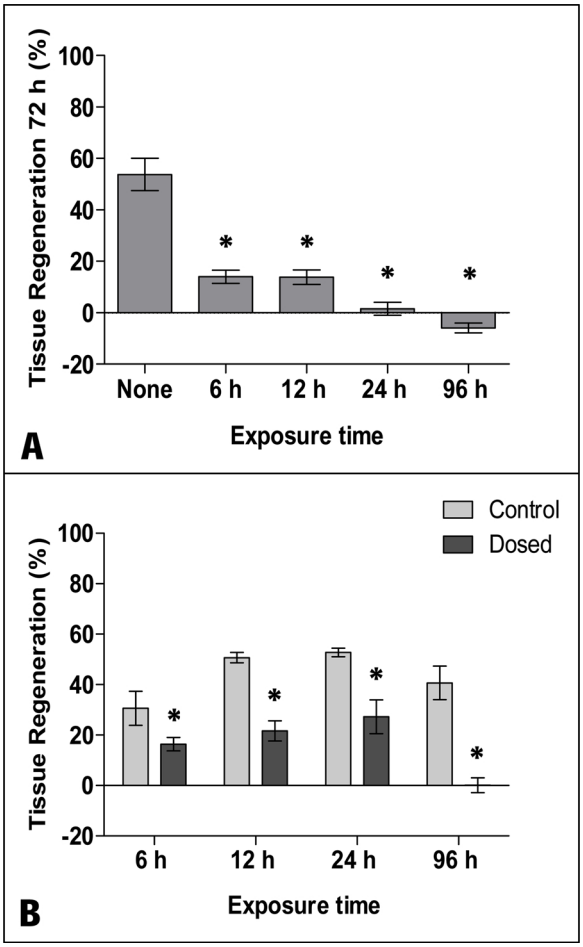


**Fig. 7.** Percent tissue regeneration for *Pocillopora damicornis* fragments after short-term HEWAF exposures (189-266 µg/L tPAH50, refreshed every 12 h). Panel A: tissue regeneration for cut fragments exposed to HEWAF. At 72 h, all treatments had significantly reduced (*) tissue regeneration when compared to the artificial seawater control ($p < 0.0001$). Fragments exposed for 96 h did not recover to the level of the controls by experiment termination (168 h post treatment). Panel B: Results of intact fragments exposed to HEWAF (cut and placed in fresh artificial seawater). Dosed and control treatments for each exposure time were imaged when controls for each treatment duration reached 30-50 % tissue regeneration (72-84 h post exposure). All dosed fragments exhibited significantly reduced ($p < 0.05$) tissue regeneration when compared to the controls (indicated by *).

et al., 2006), thus inhibiting cell membrane biosynthesis. Peters et al. (1981) observed that crude oil exposure impaired development of reproductive tissues and resulted in atrophied muscle bundles, further evidence that hydrocarbon exposure negatively impacts coral tissue growth. Combined with a possible reduction in fixed carbon availability or energy reserves from photosynthetic processes, we would expect oil-exposed corals to have greatly decreased capacity for tissue regeneration compared to unexposed corals. Furthermore, our study did not incorporate natural sunlight (with UV), thus we again emphasize that our reported toxicity threshold for this assay may represent a conservative value.

### 4.2. Time-dependent toxicity of Louisiana sweet crude oil to coral

To examine the effect of exposure duration, coral fragments were challenged with a single concentration HEWAF load over three short time periods (6 – 24 h), representing possible scenarios during an oil spill. We also included one longer exposure of 96 h for comparison to the 96 h dose-response experiment. Corals in the 6 h treatment duration were exposed in total darkness, representing the most conservative estimate of oil effects. We selected a median oil load (62.5 mg/L) from the 96 h dose-response experiment, resulting in environmentally relevant tPAH50 concentrations. PAH loss is relatively rapid due to volatilization or degradation, thus at 6 h, the tPAH50 geometric mean of fresh and spent treatment solutions was higher than at 12 h. For example, our calculated tPAH50 dose for the 6 h crude oil exposure was 266 µg/L, while the 12 h duration resulted in an exposure of 189 µg/L tPAH50. Treatments longer than 12 h were dosed repeatedly (static renewal) at 189 µg/L tPAH50.

We found that 12 h and 96 h durations of HEWAF exposure significantly affected photosynthetic quantum yield in *P. damicornis* symbionts. We did not observe any significant effect to chlorophyll fluorescence with either the 6 h or 24 h oil exposure. Variable numbers of symbionts in each coral fragment or varying amounts of chlorophyll *a* in each algal cell may explain these results, especially if 189 µg/L tPAH50 is at the lower limit for chlorophyll fluorescence effects. Additionally, the timing of the 6 h oil exposure may have resulted in symbiont recovery prior to the PAM fluorometry evaluation, particularly since this treatment was not exposed to light and oil, concurrently.

Recovery of chlorophyll *a* fluorescence was observed within 24 h after placement in fresh ASW for the fragments exposed to oil for 12 h and coral fragments in the 96 h exposure recovered after 34 h in ASW, demonstrating resilience of *P. damicornis* algal symbionts to shorter-term oil exposures. Similar quick recoveries of photosynthetic effects have been observed with placement in fresh seawater following short-term (8 – 48 h) hydrocarbon exposures (Cook and Knap, 1983; Jones and Heyward, 2003). Cook and Knap (1983) postulated that had damage occurred to cellular membranes, it would take much longer to recover and proposed that the most likely explanation of the decrease in photosynthetic output was due to interference with enzymatic processes. They did not measure chlorophyll or chlorophyll fluorescence, however, and admitted that the loss and resynthesis of chlorophyll *a* could be another likely explanation. Jones and Heyward, 2003 observed reduced chlorophyll fluorescence (Fv/Fm) from freshly-isolated symbionts ($1 \times 10^7$ cells/mL) exposed to metal- and hydrocarbon-contaminated water and gradual recovery was observed for the algal cells following placement in fresh seawater. The results from our experiments and these works support a theory of oil destruction of photosynthetic pigments. Further studies should incorporate chlorophyll measurements and enumeration of algal symbionts to provide a more definitive answer to this question.

*Pocillopora damicornis* tissue integrity and tissue color were not significantly affected by 6 – 24 h oil treatments. Timing of the health data collection (2:00 pm daily, mid-light cycle) did not coincide with HEWAF exposures for the 6, 12 and 24 h treatments, which may have resulted in underreported effects. Additionally, reduction in tissue color scores for all *P. damicornis* fragments over time may have resulted in the observed 'recovery' of the 96 h treatment duration after 209 h (Fig. 6, Panels B, E).

Polyp behavior was significantly affected by oil exposure for all treatment durations and was the principal driver of decreased health scores. This was not unexpected, since polyp retraction has been reported following coral exposure to various types of hydrocarbons (DeLeo et al., 2016; Reimer, 1975; Renegar et al., 2016; Ruis-Ramos et al., 2017). Recovery for the 6 – 24 h treatments was within 53 h (Fig. 6, Panel C). Coral fragments subjected to the 96 h HEWAF exposure exhibited severe polyp retraction at 89 h, mirroring effects noted in the 96 h dose-response experiment, and polyp retraction continued throughout the recovery period. As mentioned above, bioaccumulation of hydrocarbons has been reported for oil-exposed corals (Burns and Knap, 1989; Jafarabadi et al., 2018; Ko et al., 2014; Peters et al., 1981; Sabourin et al., 2013) and hydrocarbons can remain in coral tissues weeks after placement in clean seawater (Peters et al., 1981). The continued polyp retraction for the longest oil exposure duration may result from PAH accumulation in the lipophilic coral polyp tissues.

Polyp behavior provided a key visual clue to decreasing coral health and this health metric took days to recover to the level of the controls once the stressor was removed.

The ability to regenerate tissue over wounded areas was inhibited following 6 h of crude oil exposure (266 μg/L tPAH50) to pre-wounded *P. damicornis* fragments, demonstrating that significant health impacts do occur with short-term oil exposures. The lower tPAH50 dose (189 μg/L tPAH50) represented in the 12 − 24 h crude oil exposures also impeded coral tissue regeneration. Recoveries for all short-term exposures were delayed for 48 − 84 h after transferring fragments to fresh ASW. Complete recovery did not occur following 7 days in fresh ASW for the fragments treated with crude oil HEWAF for 96 h. This result is not unprecedented: Johannes et al. (1972) observed complete tissue breakdown for several coral species exposed directly to crude oil for 1.5 h, with no signs of regeneration on the oil-affected parts up to 4 weeks later. It is not clear if a longer recovery time would result in total recovery for these fragments, or if eventual death would occur. While there has been a recent report of polyp rejuvenescence in presumably totally 'dead' corals following a bleaching event (Kersting and Linares, 2019), significant coral tissue loss from oil exposure may be more difficult to repair, since bioaccumulated oil may continue to alter normal cellular processes long after the environmental exposure has ended.

Significantly decreased tissue regeneration was observed for intact fragments exposed to moderate tPAH50 levels and wounded in the recovery phase, indicating that oil is not simply affecting abraded tissue. This is further evidence that crude oil has effects at the cellular or subcellular level as detailed in section 4.1.3. The implications of oil damage to corals are lowered resistance to disease or predation and possible colony death, with increased negative impacts predicted for corals with lesions prior to exposure.

## 5. Conclusion

Following the DWH oil spill, measured tPAH50 concentrations in the 0 − 10 m depth (shallow water coral habitat) ranged from undetectable to over 100 μg/L, with the highest concentration observed in surface waters (0 − 1 m), 10–20 miles northwest of the wellhead (Boehm et al., 2016). Crude oil was released from the site for nearly three months, significantly damaging natural resources across many species extending hundreds of kilometers from the release site. Total PAH50 concentrations in our study were between 12 and 945 μg/L, with coral and zooxanthellae effects well within reported environmental levels for this event. The toxic responses of *P. damicornis* to the 96 h oil exposure are in the same range (low ppb) found for other marine organisms such as fish, shrimp and echinoderms (Esbaugh et al., 2016; Hemmer et al., 2011; Incardona et al., 2014; Neff et al., 2000; Stieglitz et al., 2016). We emphasize that care should be taken when comparing studies, however, since the varying analyte composition in different oil types may result in altered toxicity profiles. This is important especially when using the data in risk assessments. We note that our study results likely underestimate oil effects due to the omission of UV and the nature of the HEWAF (containing small droplets, which may not be bioavailable). Our work shows that short exposure times with moderate tPAH50 levels can negatively affect *P. damicornis* and its symbionts, providing evidence that both acute and longer-term (chronic) PAH exposures in the ppb range can impair coral health. Finally, we demonstrated that coral tissue regeneration is a very sensitive indicator of petroleum hydrocarbon exposure. With appropriate controls, wound healing could be implemented as part of a field assessment to determine impacts of oil exposure to corals.

We have limited our initial investigation to a single species and life stage and have used standardized laboratory settings (e.g., artificial light without UV, artificial seawater, etc). Photo-enhanced oil toxicity has been reported for several species of marine organisms (Alloy et al., 2016, 2015; Boese et al., 1998; Newsted and Giesy, 1987; Oris and Giesy (1987); Sweet et al., 2017) and coral larvae (Negri et al., 2016; Nordborg et al., 2018), however. We would expect that under field conditions (e.g., UV light) that oil toxicity thresholds for *P. damicornis* would be even lower than those reported in this work. Further studies should include additional species representing other distinctive morphologies, environmentally relevant levels of UV, as well as gametes or larvae, as early life stages often exhibit higher sensitivity to xenobiotic stressors.

## NOAA disclaimer

The scientific results and conclusions, as well as any opinions expressed herein, are those of the author(s) and do not necessarily reflect the views of NOAA or the Department of Commerce. The mention of any commercial product is not meant as an endorsement by the Agency or Department.

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## CRediT authorship contribution statement

**Lisa A. May:** Conceptualization, Methodology, Validation, Investigation, Data curation, Supervision, Writing - original draft, Writing - review & editing. **Athena R. Burnett:** Investigation, Formal analysis, Visualization. **Carl V. Miller:** Conceptualization, Methodology, Validation, Investigation, Resources. **Emily Pisarski:** Methodology, Validation, Investigation, Writing - original draft. **Laura F. Webster:** Investigation. **Zachary J. Moffitt:** Investigation, Resources. **Paul Pennington:** Software, Validation, Formal analysis, Visualization, Writing - original draft. **Edward Wirth:** Resources, Data curation, Writing - review & editing. **Greg Baker:** Conceptualization, Resources. **Robert Ricker:** Conceptualization, Resources. **Cheryl M. Woodley:** Conceptualization, Methodology, Resources, Project administration, Funding acquisition, Writing - review & editing.

## Declarations of Competing Interest

None.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105454.

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