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Toxicopathological Effects of the Sunscreen UV Filter, Oxybenzone (Benzophenone-3), on Coral Planulae and Cultured Primary Cells and Its Environmental Contamination in Hawaii and the U.S. Virgin Islands

C. A. Downs[1] · Esti Kramarsky-Winter[2,3] · Roee Segal[2] · John Fauth[4] · Sean Knutson[5] · Omri Bronstein[2] · Frederic R. Ciner[1] · Rina Jeger[3] · Yona Lichtenfeld[6] · Cheryl M. Woodley[7,8] · Paul Pennington[8] · Kelli Cadenas[9] · Ariel Kushmaro[3] · Yossi Loya[2]

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Abstract Benzophenone-3 (BP-3; oxybenzone) is an ingredient in sunscreen lotions and personal-care products that protects against the damaging effects of ultraviolet light. Oxybenzone is an emerging contaminant of concern in marine environments—produced by swimmers and municipal, residential, and boat/ship wastewater discharges. We examined the effects of oxybenzone on the larval form (planula) of the coral *Stylophora pistillata,* as well as its toxicity in vitro to coral cells from this and six other coral species. Oxybenzone is a photo-toxicant; adverse effects are exacerbated in the light. Whether in darkness or light, oxybenzone transformed planulae from a motile state to a deformed, sessile condition. Planulae exhibited an increasing rate of coral bleaching in response to increasing concentrations of oxybenzone. Oxybenzone is a genotoxicant to corals, exhibiting a positive relationship between DNA-AP lesions and increasing oxybenzone concentrations. Oxybenzone is a skeletal endocrine disruptor; it induced ossification of the planula, encasing the entire planula in its own skeleton. The LC_{50} of planulae exposed to oxybenzone in the light for an 8- and 24-h exposure was 3.1 mg/L and 139 μg/L, respectively. The LC_{50}s for oxybenzone in darkness for the same time points were 16.8 mg/L and 779 μg/L. Deformity EC_{20} levels (24 h) of planulae exposed to oxybenzone were 6.5 μg/L in the light and 10 μg/L in darkness. Coral cell LC_{50}s (4 h, in the light) for 7 different coral species ranges from 8 to 340 μg/L, whereas LC_{20}s (4 h, in the light) for the same species ranges from 0.062 to 8 μg/L. Coral reef

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✉ C. A. Downs
cadowns@haereticus-lab.org

1 Haereticus Environmental Laboratory, P.O. Box 92, Clifford, VA 24533, USA

2 Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, 69978 Tel Aviv, Israel

3 Avram and Stella Goldstein-Goren Department of Biotechnology Engineering and the National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, 84105 Beer Sheva, Israel

4 Department of Biology, University of Central Florida, 4000 Central Florida Boulevard, Orlando, FL 32816-2368, USA

5 Pacific Biosciences Research Center, University of Hawaii, Honolulu, HI 96822, USA

6 Department of Life Sciences, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel

7 Hollings Marine Laboratory, U.S. National Oceanic & Atmospheric Administration, 331 Ft. Johnson Rd., Charleston, SC 29412, USA

8 Center for Coastal Environmental Health and Biomolecular Research, U.S. National Oceanic & Atmospheric Administration, 219 Ft. Johnson Rd., Charleston, SC 29412, USA

9 National Aquarium, 501 East Pratt Street, Baltimore, MD 21202, USA

contamination of oxybenzone in the U.S. Virgin Islands ranged from 75 μg/L to 1.4 mg/L, whereas Hawaiian sites were contaminated between 0.8 and 19.2 μg/L. Oxybenzone poses a hazard to coral reef conservation and threatens the resiliency of coral reefs to climate change.

Oxybenzone (BP-3; benzophenone-3; 2-hydroxy-4-methoxphenyl phenylmethanone; CAS No. 131-57-7) often is used as an active ingredient in sunscreen lotions and personal-care products, such as body fragrances, hair-styling products, shampoos and conditioners, anti-aging creams, lip balms, mascaras, insect repellants, as well as dishwasher soaps, dish soaps, hand soaps, and bath oils/salts (CIR 2005; http://www.goodguide.com/ingredients/184390-oxybenzone). BP-3 and other benzophenone derivatives often are found as contaminants in boating, residential, and municipal wastewater effluents and are considered "emerging environmental contaminants of concern" by the U.S. Environmental Protection Agency (Eichenseher 2006; Richardson 2006, 2007; Blitz and Norton 2008; Gago-Ferrero et al. 2011; Kameda et al. 2011; Rodil et al. 2012; Aquero et al. 2013).

Between 6000 and 14,000 tons of sunscreen lotion, many of which contain between 1 and 10 % BP-3, are estimated to be released into coral reef areas each year, putting at least 10 % of the global reefs at risk of exposure, and approximately 40 % of coral reefs located along coastal areas at risk of exposure (Shaath and Shaath 2005; UNWTO 2007; Danovaro et al. 2008; Wilkinson 2008). In Okinawa, BP-3 levels on coral reefs that were 300–600 m away from public swimming beaches ranged from 0.4 to 3.8 pptrillion (Tashiro and Kameda 2013); in South America, sediments near coral communities/reefs contained BP-3 concentrations between 54 and 578 pptrillion (Baron et al. 2013). Schlenk et al. (2005) discovered through a Toxicity Identification Evaluation that BP-3 was unequivocally identified as the source of estrogenic activity in marine sediments near wastewater outfalls. Although the half-life in seawater is several months, BP-3 can act as a pseudo-persistent pollutant; its contamination of a site may be constantly renewed, resulting in ecological receptors experiencing persistent exposure (Vione et al. 2013). Concerns regarding the adverse impacts of exposure to BP-3 on coral reefs and other marine/aquatic ecosystems have led to either banning oxybenzone-containing products in marine-managed areas (e.g. Mexico's marine ecoparks; Xcaret 2007; Xel-há 2007) or public relations campaigns by management agencies to encourage reduction of environmental contamination of sunscreen lotions by swimmers (e.g. "Protect Yourself, Protect the Reef" Bulletin U.S. NPS 2012).

BP-3 exhibits a number of toxicological behaviors ranging from the molecular level to multi-organ system pathologies (Gilbert et al. 2012). Benzophenones, including BP-3, are documented mutagens that increase the rate of damage to DNA, especially when exposed to sunlight (Popkin and Prival 1985; Zeiger et al. 1987; Knowland et al. 1993; NTP 2006). BP-3 produced a positive mutagenic response by inducing the *umu* operon (genotoxicity assay Nakajima et al. 2006). Benzophenones, and especially BP-3, either can act directly as genotoxicants or become genotoxicants by bioactivation via cytochrome P450 enzymes (Takemoto et al. 2002; Zhao et al. 2013). The types of damage to genetic material by benzophenones include oxidative damage to DNA, formation of cyclobutane pyrimidinic dimers, single-strand DNA breaks, cross-linking of DNA to proteins, and an increase in the formation of DNA abasic sites (Cuquerella et al. 2012). Benzophenones also exhibit pro-carcinogenic activities (Kerdivel et al. 2013). BP-3 can generate reactive oxygen species, which are potential mutagens, when applied topically to the skin followed by UV light exposure (Hanson et al. 2006).

BP-3 is a reproductive toxicant whose mechanisms of action and its pathological effects are poorly characterized in various model species. In mice studies, BP-3 exposure significantly affected fecundity, as well as inducing unexplained mortality in lactating mothers (Gulati and Mounce 1997). Studies in both mice and rats demonstrated that generational exposure to BP-3 reduced body weight, increased liver (>50 %) and kidney weights, induced a 30 % increase in prostate weight, a reduction in immunocompetence, and significantly increased uterine weight in juveniles (Gulati and Mounce 1997; French 1992; Schlumpf et al. 2008; Rachon et al. 2006). In mammals, BP-3 is renowned for having estrogenic and anti-androgenic activities, causing activation of estrogen receptor proteins and inhibition of androgen receptors (Morohoshi et al. 2005; Suzuki et al. 2005; Kunz et al. 2006; Molina–Molina et al. 2008; Nashez et al. 2010). Topical application of BP-3 to the skin has been shown to be absorbed and transferred to breast milk, creating risk to breast-fed neonates (Hany and Nagel 1995). In addition, an association between exposure to benzophenones and an increased occurrence of endometriosis in women was recently found by Kunisue et al. (2012).

In fish, BP-3 actions are similar to those in mammals, causing an endocrine disruption by modulating estrogen receptor signaling pathways, inducing reproductive pathologies, and reducing reproductive fitness (Kunz et al. 2006; Coronado et al. 2008; Cosnefroy et al. 2011; Bluthgen et al. 2012). Chronic exposure to BP-3 in fish resulted in reduced egg production, induction of vitellogenin protein in males, and a significant reduction in egg hatchings (Nimrod and Benson 1998; Coronado et al. 2008). These findings raise the possibility of "gender shifts" in fish exposed to BP-3 during the entirety of their

life history or during "windows of sensitivity" (Coronado et al. 2008).

A few studies exist that have evaluated the effects of BP-3 exposure in invertebrates. In insects, BP-3 inhibited expression of the *usp* gene (ultraspiracle protein)—a protein that combines with the EcR protein to form the ecdysone receptor, which controls aspects of developmental and reproductive processes (Ozáez et al. 2013). Gao et al. (2013) found that BP-3 exposure resulted in oxidative injuries, reduced glutathione, and adversely affected cell viability in the protozoan ciliate, *Tetrahymena thermophila.*

Since the 1970s, coral reefs have been devastated on a global scale. Regional weather and climate events often are responsible for acute events of mass-mortality of coral reefs (Carpenter et al. 2008). However, the long-term causative processes of sustained demise often are locality specific (Edinger et al. 1998; Rees et al. 1999; Golbuu et al. 2008; Smith et al. 2008; Downs et al. 2011, 2012; Omori 2011). Records of coral recruitment in many areas of the Caribbean, Persian Gulf, Red Sea, Hawaiian Islands, and elsewhere have exhibited precipitous declines (Richmond 1993, 1997; Hughes and Tanner 2000; Rogers and Miller 2006; Williams et al. 2008). This is most apparent in the deterioration of juvenile coral recruitment and survival rates along coastal areas (Dustan 1977; Miller et al. 2000; Abelson et al. 2005; Williams et al. 2008). As with other invertebrate species, coral larvae (i.e., planula) and newly settled coral (i.e., recruits) are much more sensitive to the toxicological effects of pollution compared with adults (Kushmaro et al. 1997). Hence, even small impacts to larval development and survival can have significant effects on coral demographics and community structure (Richmond 1993, 1997). To manage BP-3 pollution and mitigate its effect on the ecological resilience of coral reefs, the toxicological effects of BP-3 on larval survival and development need to be characterized (Fent et al. 2010; US EPA 2012; NRC 2013).

In this study, we examined the toxicological effects of exposures to varying concentrations of BP-3 on the larval form (planula) of the scleractinian coral *Stylophora pistillata,* the most abundant coral species in the northern Gulf of Aqaba, Red Sea (Loya 1972). Many chemical pollutants affect organisms differently when exposed to light, a process known as chemical-associated phototoxicity (Yu 2002; Platt et al. 2008). Because reef-building corals are photosynthetic symbiotic organisms, and many coral species have planulae that are photosynthetically symbiotic (e.g., *S. pistillata*), we examined the effects of BP-3 exposure in planulae subjected to either darkness or to environmentally-relevant light conditions. Histopathology and cellular pathology, planula morphology, coral bleaching, DNA damage as the formation of DNA abasic lesions, and planula mortality were measured in response to BP-3 exposure. Median lethal concentration (LC_{50}), effect concentration (EC_{20}), and no observable effect concentrations (NOEC) were determined for coral planulae exposed to BP-3 in both darkness and in light. Coral planulae are a relatively difficult resource to procure for toxicological studies. Therefore, primary coral cell cultures were used in in vitro toxicological tests of BP-3 to examine their validity as a surrogate model for coral planulae in generating an effect characterization as part of an Ecological Risk Assessment. The confidence in this model was examined by comparisons of the LC_{50} results of BP-3-exposed planulae to the BP-3 LC_{50} of coral cells (calicoblasts) from adult *S. pistillata* colonies. Coral-cell toxicity testing was conducted on six other species that originate from either the Indo-Pacific or Caribbean Sea/Atlantic Ocean basins to provide in vitro data on the species' sensitivity distribution of BP-3. To determine the environmentally relevant concentration of BP-3 in seawater on coral reefs, we measured BP-3 concentrations at various locations in the U.S. Virgin Islands and the U.S. Hawaiian Islands.

Materials and Methods

Planula Collection and Toxicity Exposures

Planula collection and planula-toxicity exposures were conducted at the Inter-University Institute of Marine Sciences (IUI) in Eilat, Israel. *Stylophora pistillata* (Esper 1797) planulae were collected from the wild within the IUI designated research area by placing positively buoyant planula traps over *Stylophora* colonies measuring more than 25 cm in diameter. Permit for collection was given to Y. Loya by the Israel National Park Authority. Traps were set between 17:00 and 18:00 h, and then retrieved at 06:00 h the next morning. Planulae were inspected and sorted by 07:15 h, and toxicity exposure experiments began at 08:00 h.

Experimental design and culture conditions were based on modified (for coral) guidelines set forth in OECD (2013) and described in Downs et al. (2014). This experiment for BP-3 was conducted concurrently with the study conducted in Downs et al. (2014).

All seawater (ASW) was made artificially using Fisher Scientific Environmental-Grade water (cat#W11-4) and Sigma-Aldrich sea salts (cat#S9883) to a salinity of 38 parts per thousand at 22 °C. Benzophenone-3 (BP-3; 2-Hydroxy-4-methoxyphenyl-phenylmethanone; Aldrich cat#T16403) was solubilized in dimethyl sulfoxide (DMSO) and then diluted with ASW to generate stock solutions and exposure solutions. Solutions of BP-3 for toxicity exposures each contained 5 microliters of DMSO

per one liter and were of the following concentrations: 1 mM BP-3 (228 parts per million), 0.1 mM BP-3 (22.8 mg/L; parts per million), 0.01 mM BP-3 (2.28 mg/L; parts per million), 0.001 mM BP-3 (228 µg/L; parts per billion), 0.0001 mM (22.8 µg/L; parts per billion), and 0.00001 mM (2.28 µg/L; parts per billion). For every exposure time-period, there were two control treatments with four replicates each: (a) planulae in ASW, and (b) planulae in ASW with 5 microliters of DMSO per 1 L. There was no statistical difference between the two controls for any of the assays.

Planulae were exposed to different BP-3 concentrations during four different time-period scenarios: (a) 8 h in the light, (b) 8 h in the dark, (c) a full diurnal cycle of 24 h, beginning at 08:00 in daylight and darkness from 18:00 in the evening until 08:00 h the next day, and (d) a full 24 h in darkness. For the 24-h exposure, planulae from all treatments were transferred to new 24-well microplates with fresh ASW/BP-3 media at the end of the 8-h daylight exposure before the beginning of the 16 h dark exposure.

At the end of the 8 and 24-h time points, chlorophyll fluorescence, morphology, planula ciliary movement, and mortality were measured, while at least one planula from each replicate of each treatment was chemically preserved, and the remaining living planulae were flash frozen in liquid nitrogen for the DNA apyrimidinic (AP) site assay.

Chlorophyll Fluorescence as an Estimate of Bleaching

Chlorophyll fluorescence was measured using a Molecular Dynamics microplate fluorometer with an excitation wavelength of 445 nm and an emission wavelength of 685 nm. Fluorescence measurements were taken at the end of the 8-h light and dark periods of BP-3 exposure. All ten planulae in each replicate well were measured in aggregate. Each well was measured independently of the other wells. Justification and caveats for this assay are described in Downs et al. (2014).

DNA Abasic Lesions

DNA abasic or apurinic/apyrimidinic lesions (DNA AP sites) were quantified using the Dojindo DNA Damage Quantification Kit-AP Site Counting (DK-02-10; Dojindo Molecular Technologies, Inc.) and conducted as described in Downs et al. (2014). Four individual planulae (one from each well) from each treatment were individually assayed. Only planulae that were relatively intact were assayed, even if scored as dead. Planulae from 228 ppm BP-3 at 8 h in the light were not collected, because there were no coherent planulae.

Transmission Electron Microscopy

Transmission electron microscopy was used for tissue and cellular pathomorphology assessment on three planulae from each treatment. Methodology for this technique was described in Downs et al. (2014). At least three planula from each treatment were collected and fixed for analysis.

Coral Cell Toxicity Assay

Cultured colonies of *S. pistillata* (Esper 1797) were obtained from Exotic Reef Imports (www.exoticreefimports.com) and did not need a permit for possession. Cultured colonies of *Pocillopora damicornis* (Linnaeus 1758) was provided by the National Aquarium and did not need a permit for possession. *Montastrea annularis*, *Montastrea cavernosa* (Linnaeus 1766), and *Porites astreoides* (Lamarck 1816) were obtained from the Florida Keys National Marine Sanctuary under permit# FKNMS-2011-139. Cultured colonies of *Acropora cervicornis* (Lamarck 1816) and *Porites divaricata* (Lesueur 1821) were provided by Dr. Cheryl Woodley of the U.S. National Oceanic and Atmospheric Administration and did not need a permit for possession. Corals were maintained in glass and Teflon-plumbed aquaria in 36 ppt salinity artificial seawater (Type 1 water using a Barnstead E-Pure filter system that included activated carbon filters) at a temperature of 24 °C. Corals were grown under custom LED lighting with a peak radiance of 288 photosynthetic photon flux density µmol/m^2/s. Light Spectra ranged from 380 to 740 nm. Light was measured using a Licor 250A light meter and planar incidence sensor. Description of coral cell isolation from each species is described in Downs et al. (2010, 2014).

Exposure experiments of cells were conducted in PTFE-Telfon microplates. Cells of all species except *Acropora cervicornis* were exposed to BP-3 concentrations in cell culture media of 570 parts per trillion to 228 parts per million for 4 h in the light, whereas *Stylophora* cells also were exposed for 4 h in the dark. *Acropora cervicornis* cells were exposed to BP-3 concentrations in cell culture media of 570 ng/L (parts per trillion) to 228 mg/L (parts per million) for 4 h in the light. Lighting was from custom LED fixtures that had wavelength emissions from 390 to 720 nm with a light intensity of 295 µmol/m^2/s of photon flux density.

Viability was confirmed using the trypan blue exclusion assay. There were four replicate wells with cells per treatment. Duplicate aliquots of cells from each replicated wells were collected into a microcentrifuge tube, centrifuged at 300×*g* for 5 min, and the supernatant aspirated. Cells were gently resuspended in culture media that contained 0.5–1.5 % (w/v) of filtered trypan blue (Sigma-

Aldrich, cat#T6146), and incubated for 5 min. Viable versus dead cells were counted using a modified Neubauer hemocytometer (Hausser-Levy Counting Chamber).

Sampling and analysis of benzophenones in seawater samples via gas chromatography-mass spectrometry (GC–MS) and liquid chromatography-mass spectrometry (LC–MS). Dichloromethane, methanol, acetone are pesticide-grade solvents (Fisher Scientific). Analytical standards were purchased from Sigma Aldrich and included: Benzophenone (cat# B9300), Benzhydrol (cat#B4856), 4-hydroxybenzophenone (cat#H20202), 2-hydroxy-4-methoxy benzophenone (cat#H36206), 2,4-dihydroxy benzophenone (cat# 126217), 2-2′-dihydroxy-4-methoxy benzophenone (cat# 323578), 2,3,4-trihydroxy benzophenone (cat# 260576), 2,2′,4,4′-tetrahydroxy benzophenone (cat#T16403). Internal standard solutions (phenanthrene-d10 and chrysene-d12) were purchased from AccuStandard Inc. (New Haven, CT).

Field personnel collecting samples were subject to an Alconox Liqui-Nox detergent decontamination immediately before entering the sampling site and did not apply any sunscreen lotion or nonorganic personal-care products to their body for at least 21 days before sampling. Between 100 and 500 mL of seawater were collected approximately 35 cm below the surface of the water into EPA-certified clean, amber jars. In the field, water samples were extracted using Phenomenex C18 solid phase extractions columns that were first activated with methanol. All columns were capped and then shipped and stored frozen at −80 °C or colder.

Extraction of analytes from seawater samples collected in the U.S. Virgin Islands (under a U.S. National Park Service permit, STT-045-08) followed the methodology described in Jeon et al. (2006). Seawater samples were collected using precleaned 1-L amber glass bottles with Teflon lined lids (I-Chem, 300 series, VWR). Seawater samples were extracted using C-18E cartridges (500 mg, 6 mL Phenomenex Inc.) on a vacuum manifold (Phenomenex Inc.). Cartridges were conditioned with 5 mL of methanol and then 5 mL of water, after which the seawater samples were then added to the column. Following extraction, the cartridges were dried for 10 min, capped, and frozen until processed. The cartridges were eluted with 2 mL of acetone followed by 2 × 5 mL dichloromethane. The extracts were evaporated to dryness under a gentle stream of nitrogen. Then, 50 µL of MSTFA (*N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide, Sigma-Aldrich) was added, capped, vortexed for 30 s, and heated at 80 °C for 30 min. Extracts were transferred to gas chromatography vials with a rinse step to a final volume of 1 mL and the internal standard was added. Percentage recovery for all 8 target analytes using this method with seawater was >95 %.

Seawater samples from Hawaii were collected using precleaned one liter amber glass bottles with Teflon lined lids (I-Chem, 300 series, VWR). Samples were extracted using C-18E cartridges (500 mg, 6 mL Phenomenex Inc.) on a vacuum manifold (Phenomenex Inc.). Cartridges were conditioned as indicated in the previous paragraph and eluted with 5 mL of methanol. For LC–MS analysis, samples were run on an AB_SCIEX 5500 QTRAP Triple Quadrupole Hybrid Linear Ion Trap Mass Spectrometer with a Spark Holland Symbiosis HPLC for analytical separation. The analytes were measured with MRM (multiple reaction monitoring) followed by switching to ion trap functionality (Q3- LIT) to confirm the fragmentation pattern of the MRMs. The source was set at 700 °C and the gasses were set to 60 arbitrary units of nitrogen. The curtain gas was set at 45 arbitrary units, and all MRMs were optimized using infusion based introduction of analytical standards. Analytical separation was performed using a Phenomenex Hydro RP 4.6 × 50 2.6 µm particle size stationary phase, with the mobile phase composed of methanol and water with the addition of 0.1 % formic acid and 5 mM of ammonium acetate in both phases. The flow rate was set at 0.9 mL per min, and a ballistic gradient and re-equilibration was run over 5 min. Percentage recovery for target analytes was >85 %, Limit of Detection was 100 pptrillion, and Quantitative Limit of Measurement was 5 ppbillion (µg/L).

Statistical Methods

OECD (2006) was used as a guidance document for our approach in the statistical analysis of the data. To address different philosophies and regulatory criteria, Effect Concentration response (EC_{20} and EC_{50}) and median Lethal Concentration response (LC_{50}) were determined using three initial methods: PROBIT analysis (Finney 1947), linear or quadratic regression (Draper and Smith 1966), and spline fitting (Scholze et al. 2001). Data were analyzed using linear or quadratic regression and PROBIT methods individually for each experiment, based on model residuals being random, normally distributed, and independent of dosing concentrations (Crawley 1993, Fig. 5.1), as well as having good fit, statistically significant, and biologically interpretable regressors (Agresti 2002; Newman 2013). Spline fitting did not meet these criteria. In several analyses, BP-3 concentrations as $\log_{10}(x + 1)$ were transformed to conform to model assumptions.

Data were tested for normality (Shapiro–Wilk test) and equal variance. When data did not meet the assumption of normality and homogeneity, the no-observed-effect concentration (NOEC) was determined using Kruskal–Wallis one-way analysis of variance, using Dunnett's Procedure (Zar 1996) to identify concentrations whose means differed

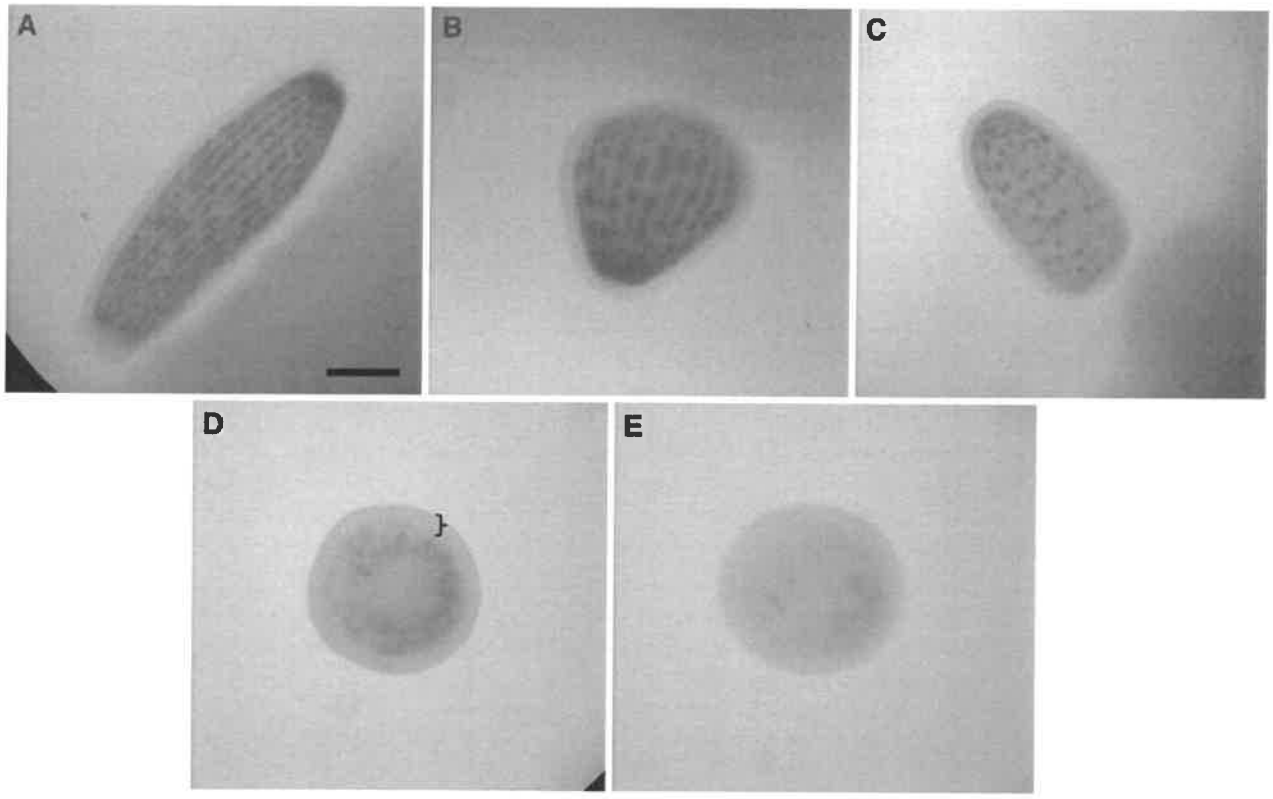

Fig. 1 *Stylophora pistillata* planulae exposed to various treatments of benzophenone-3 (BP-3). **a** Control planula exposed for 8 h in light. **b** Planula exposed to 22.8 parts per billion (μg/L) BP-3 for 8 h in the light. **c** Planula exposed to 228 parts per billion (μg/L) BP-3 for 8 h in the light. **d** Planula exposed to 2.28 parts per million (mg/L) BP3 for 8 h in the light. **e** Planula exposed to 28.8 parts per million (mg/L) BP3 for 8 h in the light. *Scale bar* is 0.5 mm

significantly from the control (Newman 2013). When variances among treatments were heterogeneous, we verified these results using a Welch ANOVA. In cases where responses were homogeneous within the control treatment (i.e., all planulae survived) or another concentration (i.e., all planulae died or were deformed), the Steel Method (Steel 1959) was substituted, which is the nonparametric counterpart to Dunnett's Procedure (Newman 2013). Four replicates of each experimental concentration provided good statistical power for parametric analyses, but it is cautioned that the relatively small sample size for the nonparametric Steel Method (Steel 1959) made results of this test less powerful. To facilitate comparisons among other treatment means, figure legends include results of Newman–Keuls Method post hoc test, which compares each concentration to all others.

Parametric (Pearson's r) or nonparametric (Spearman's ρ) regression analyses were used to determine the relationship between mortality of coral planulae and coral cells. Coral planulae are available only immediately after spawning and a strong association between these two responses would allow mortality of coral cells to serve as a surrogate for this reproductive response. JMP version 9.0 or 10.0 (SAS Institute, Inc., Cary, NC), SAS version 9.3 and SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA) were used for analyses.

Results

Toxicopathology

Planulae under control conditions have an elongated, "cucumber-like" morphology with organized rows of zooxanthellae-containing gastrodermal cells running from the aboral pole to the oral pole (Fig. 1a; "brown dots" in the rows are individual zooxanthella cells). Normal planulae are in near-constant motion, being propelled by cilia that cover the elongated body. Within the first 4 h of exposure of planulae to BP-3 in both light and darkness, planulae showed a significant reduction in ciliary movement and the morphology had significantly changed from the elongated form to a deformed "dewdrop" (Fig. 1b). At 228 μg/L BP-3, planulae contain noticeably less zooxanthellae (brown spots) indicative of "bleaching" (Fig. 1c). The mouth of the planula at the oral pole began to increase three- to fivefold in diameter at the end of the 8-h exposure

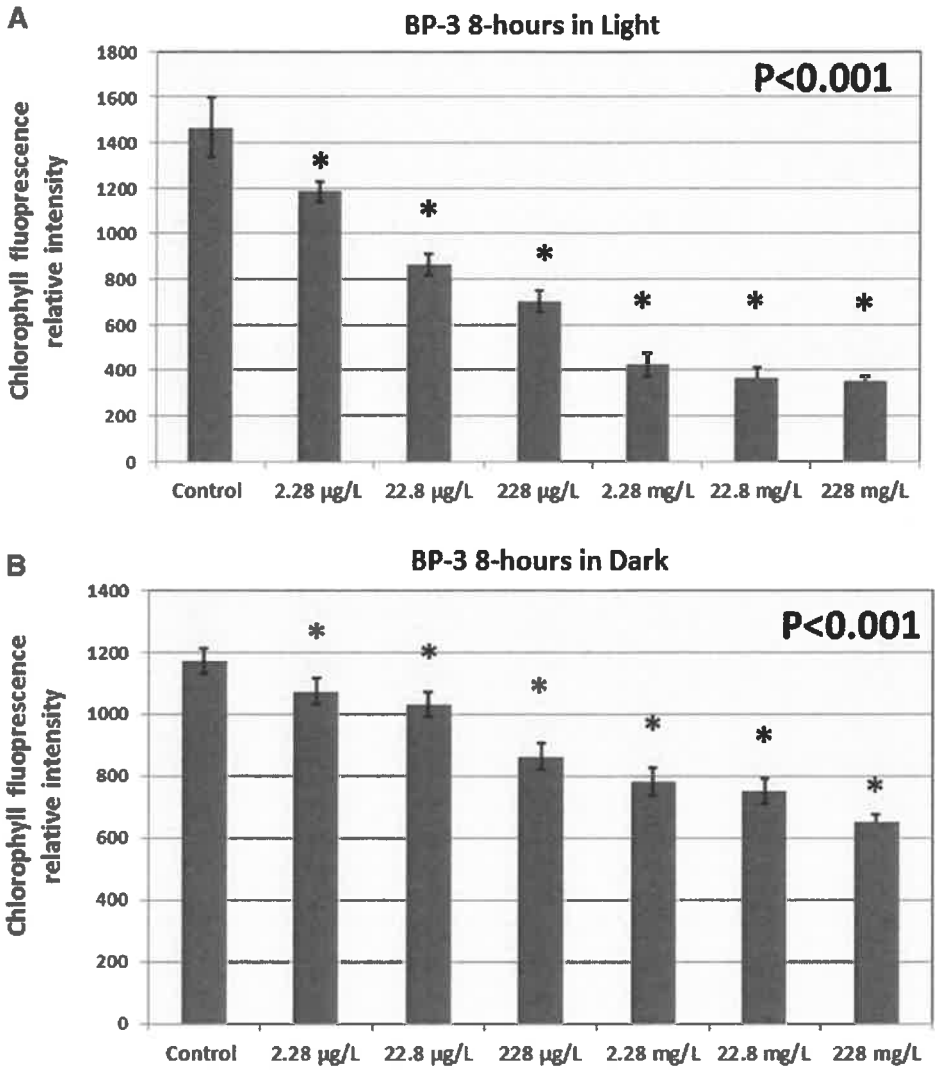

Fig. 2 Relative chlorophyll fluorescence emission at 685 nm with excitation at 445 nm of planulae of *Stylophora pistillata* exposed to various treatments of benzophenone-3 (BP-3). *Bars* show treatment means with whiskers representing ±1 standard error of the mean. $N = 4$ replicates per treatment. **a** Planulae exposed to various BP-3 concentrations for 8 h in the light. Treatment means with *different letters* differed significantly from the control at $\alpha = 0.05$, based on Kruskal–Wallis one-way analysis of variance on ranks followed by a Dunnett's Method post hoc test against a control. **b** Planulae exposed to various BP-3 concentrations for 8 h in the dark. Treatment means with different *superscript letters* differed significantly from the control at $\alpha = 0.05$, based on one-way analysis of variance followed by a Dunnett's Method post hoc test against control

(Fig. 1d). By the end of the 8 h of exposure for all BP-3 concentrations, the oral pole was recessed into the body in deformed planulae (Fig. 1b) and the epidermis of all the deformed planulae took on a white opaque hue. For planulae exposed to the higher concentrations of BP-3, it was apparent that the epidermal layer had lost its typical transparency and become opaque (Fig. 1, bracket indicates opaqueness of epidermal layer).

At the end of the 8-h exposure, all planulae exposed to all of the concentrations of BP-3 became sessile. Additionally, there was a positive relationship between exposure to increasing concentrations of BP-3 and planula bleaching (Figs. 1a–e, 2). Bleaching is the loss of symbiotic dinoflagellate zooxanthellae, photosynthetic pigments, or both. Chlorophyll fluorescence as an indicator of the concentration of chlorophyll *a* pigment corroborated these visual observations; exposure to BP-3, whether in light or darkness, caused planulae to bleach (Fig. 2). The Lowest Observable Effect Concentration for inducing chlorophyll-defined bleaching is 2.28 µg/L in the light ($P < 0.001$, Dunnett's Method) and 22.8 µg/L in the dark ($P < 0.01$, Dunnett's Method).

Normal planulae have four layers of organization. At the surface of the planula is the epidermis (Fig. 3a–c). The outer aspect of the epidermis has densely packed ciliated cells (Fig. 3a), spirocysts and nematocysts/blasts (Fig. 3b), and cells containing chromogenic organelles. Between the epidermis and the gastrodermal tissue layers is the mesoglea (Fig. 3c–d). Within the gastrodermal tissue are cells that contain symbiotic dinoflagellate zooxanthellae within an intracellular vacuole (Fig. 3e). Figure 3e depicts a healthy morphology, with the presence of starch granules, coherent chloroplasts, and the presence of a pyrenoid body that interfaces with chloroplasts. Figure 3f illustrates the integrity of chloroplasts (cp) within the dinoflagellate, especially the structure of the tri-partite rows of the

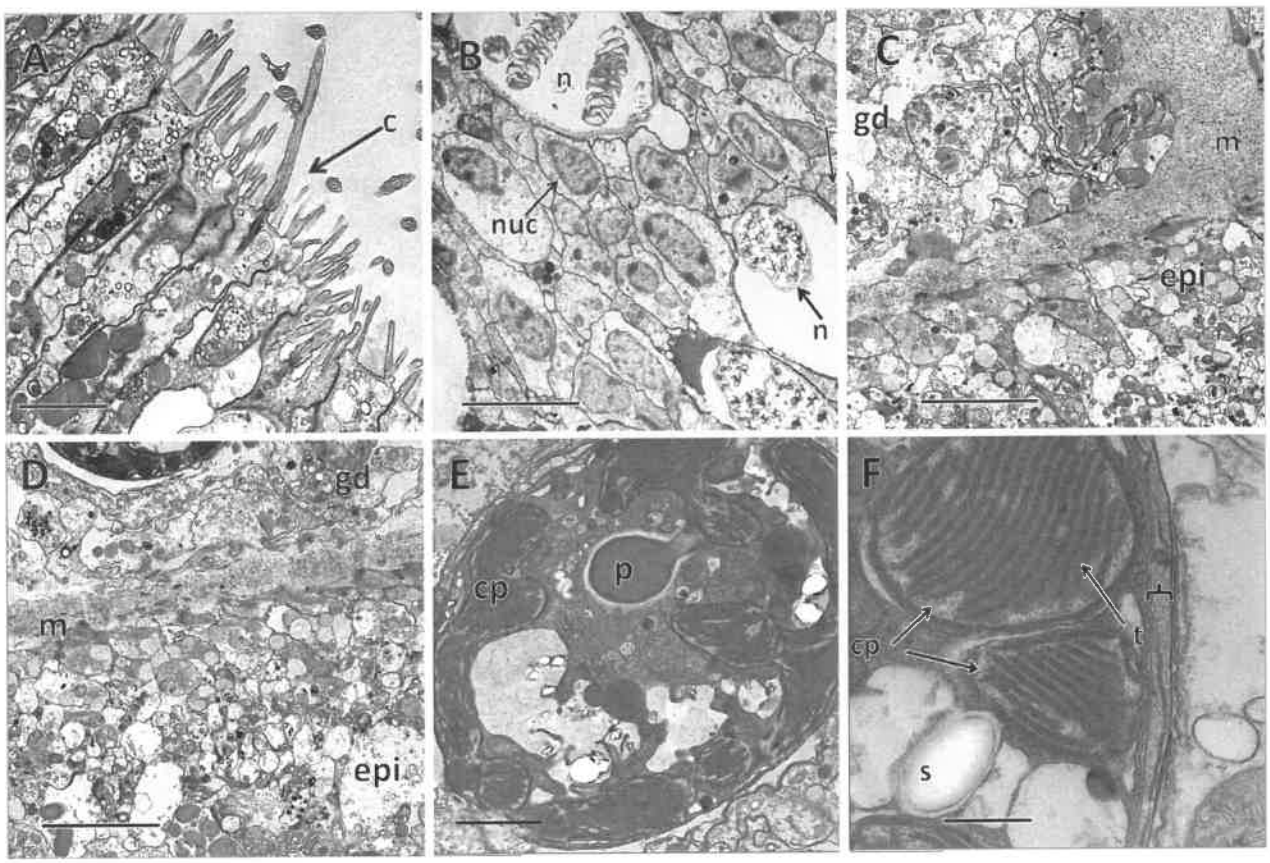

Fig. 3 Transmission electron microscopy of *Stylophora pistillata* planula control treatment. **a** Epidermal surface, indicating the presence of functional cilia (c) and tightly adjoined epidermal cells; *bar* indicates 2000 nm. **b** Epidermal surface indicates intact nematocysts (n) and nuclei (nuc); *bar* indicates 5000 nm. **c** Mesoglea (m) demarks the epidermal tissue (epi) from the gastrodermal tissue (gd); *bar* indicates 5000 nm. **d** Micrograph indicates the interface of the gastroderm (g), mesoglea (m), and epidermis (epi); *bar* indicates 5000 nm. **e** Zooxanthella in the gastrodermal tissue of planula, indicating the presence of intact chloroplasts (cp) and pyrenoid body (p). Notice the absence of a vacuolar space between the coral vacuolar membrane and the thecal plates/membrane of the zooxanthella; *bar* indicates 2000 nm. **f** Close-up of cytosolic structure of zooxanthella. Chloroplasts (cp) exhibit intact chloroplastic membrane and coherent, parallel rows of thylakoid membranes. *Bracket* (]) indicates the absence of vacuolar space between the coral vacuolar membrane and the zooxanthella's thecal plate/membrane; *bar* indicates 500 nm

thylakoid (t) membranes. Dinoflagellates from control planulae contained an abundance of starch granules (S), as well as the absence of vacuolated space between the dinoflagellate's thecal plate and the host's symbiophagic membrane (indicated by "{"; Fig. 3f).

Transmission electron microscopy of planula exposed to 288 parts per billion BP-3 for 8 h in the light (Fig. 4) showed that the planulae experienced catastrophic tissue lysis and cellular degradation in both the epidermis and gastrodermis, as well as partial collapse of the mesoglea (Figs. 3 vs. 4). At the surface of the epidermis, there was a complete loss of ciliated cells (Fig. 4a). The development and extent of cell death and tissue deterioration was greatest at the surface of the epidermis and became less pronounced at the center of the planula. In the middle area of the epidermal tissue, between the outer surface of the epidermis and its boundary with the mesoglea, the incidence of autophagic cell death became more pronounced (Fig. 4b; Tsujimoto and Shimizy 2005; Samara et al. 2008). Individual cells were dense with autophagic bodies, and many of the nuclei exhibited delamination of the nuclear bilayer membrane and vacuolization of the inner nuclear membrane containing chromatin (Fig. 4c; "}" indicates vacuolization; Eskelinin et al. 2011). None of the nuclei observed in the micrographs exhibited any signs of apoptosis, such as condensation of chromatin (Kerr et al. 1972; White and Cinti 2004; Taatjes et al. 2008). Specialized cells, such as spirocysts, also exhibited deterioration (Fig. 4d). The mesoglea exhibited structural deterioration; this vascular space contained an abundance of debris, including detached cells (Fig. 4e). The gastrodermis also exhibited extensive trauma (Fig. 4e–g). Many gastrodermal cells exhibited considerable dense autophagic bodies (Fig. 4f), although there were a few instances of

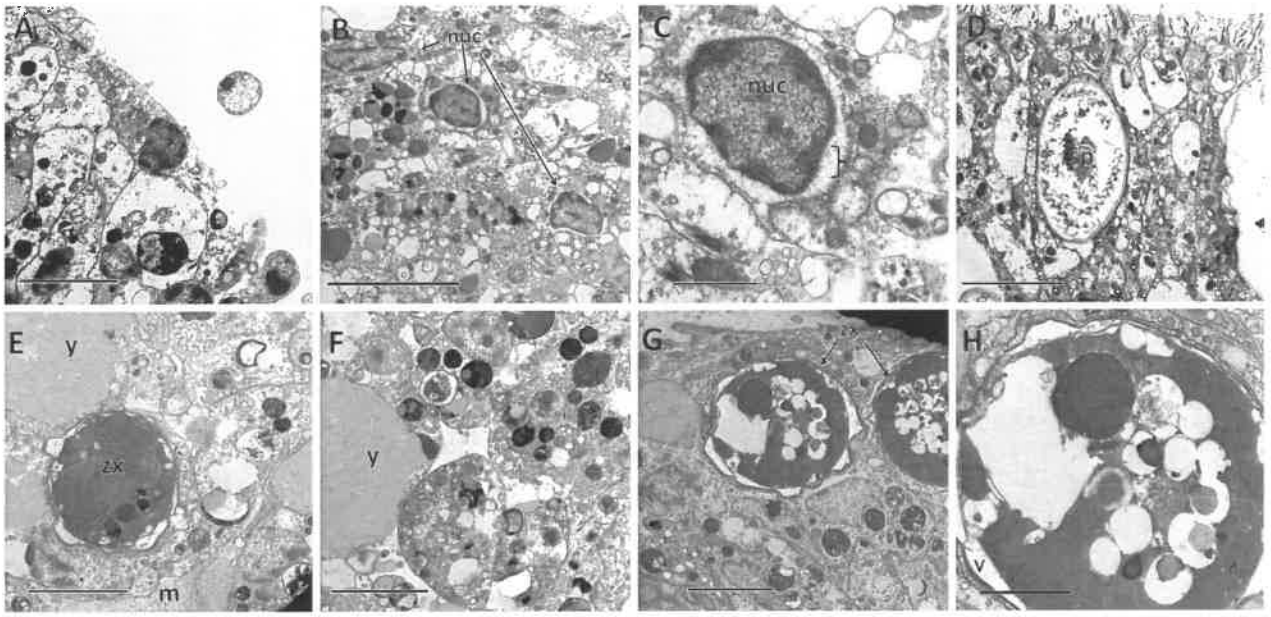

Fig. 4 Transmission electron microscopy of *Stylophora pistillata* planula exposed to 228 parts per billion (μg/L) benzophenone-3 for 8 h in the light. **a** Surface of the epidermal layer; indicating a lack of cilia and cells dying either via necrosis or autophagic cell death; *bar* indicates 5000 nm. **b** Epidermal tissue where cells exhibit an abundance of vacuolated bodies, especially the presence of vacuolated nuclei (nuc); *bar* indicates 5000 nm. **c** Magnification of vacuolated nuclei (nuc) that completely lacks nuclear blebbing (a sign of apoptosis). "}" indicates vacuolization of delaminated nuclear double membrane; *bar* indicates 1000 nm. **d** Epidermal layer with vacuolated ciliated cells, spirocysts (sp) and nematocysts; *bar* indicates 5000 nm. **e** Micrograph depicts intersection of mesoglea (m) and gastrodermal tissue containing both zooxanthella (zx) gastrodermal cells and yolk (y); *bar* indicates 5000 nm. **f** Epidermal tissue adjacent to yolk exhibits extensive autophagic vacuolization; *bar* indicates 5000 nm. **G** Gastrodermal cells containing symbiophagic zooxanthellae. Zooxanthellae have undergone extensive internal vacuolization; *bar* indicates 5000 nm. **h** Increased magnification focused on vacuolated zooxanthella, (v) indicates symbiophagic vacuole; *bar* indicates 2000 nm

nuclear autophagy. Gastrodermal cells containing symbiotic zooxanthella exhibited the early stages of symbiophagy, with vacuolization occurring around the zooxanthella (Fig. 4e–g). None of the zooxanthellae showed "normal" morphologies. They instead displayed extensive internal vacuolization, homogenization of chromatin density, and chloroplast degradation, especially of the thylakoid membranes (Fig. 4g–h).

Transmission electron microscopy of planulae exposed to 228 μg/L BP-3 for 8 h in darkness (Fig. 5) exhibited a similar gradient of cell death and tissue deterioration from the surface of the planula to its center as seen in planulae exposed to BP-3 in the light, although the progression of cellular deterioration was not as severe (Fig. 5a–h). Along the surface of the epidermal tissue layer, ciliated cells were undergoing cellular degradation (Fig. 5a). The cell layer immediately below the ciliated cells was degraded, characterized by an abundance of vacuolated bodies and loss of the plasma membrane (Fig. 5b, c). Many of the nuclei exhibited partial delamination of the bilayer nuclear membrane, but unlike the nuclei observed in planulae exposed to BP-3 in the light, vacuolization was not complete and the bilayer was still partially anchored by nuclear pores (Fig. 5b, c). Deeper into the epidermal layer, along the boundary with the mesoglea, cellular degradation persisted, especially of the spirocysts (Fig. 5d). There is an extracellular matrix that acts as a barrier between the epidermal tissue and mesoglea, and again between the gastrodermal tissue and mesoglea. Under these conditions, the integrity of the boundary layer between the epidermis and mesoglea had severely deteriorated, whereas the boundary layer between the gastrodermis and mesoglea remained intact (Fig. 5e). Within the gastrodermis, a vast majority of the cells were alive, but exhibiting signs of massive autophagy (Fig. 5f; Klionsky et al. 2012). It should be noted that there were almost no instances of delamination of the nuclear membrane in the gastrodermal cells; nuclei looked healthy (Fig. 5f). Many of the cells were dense with autophagosomic bodies, and most of the zooxanthellae were undergoing symbiophagy, as indicated by the vacuolization around the dinoflagellate cell (Fig. 5f; Downs et al. 2009). In zooxanthellae that were not significantly degraded (Fig. 5f vs. h), thylakoids exhibited a pathomorphology similar to that found in zooxanthellae of corals exposed to heat stress (32 °C) in darkness; thylakoid lamellae were diffuse (Fig. 5g; Downs et al. 2013), suggesting that the zooxanthellae were directly affected by the BP-3 exposure. In contrast to the findings of Danovaro

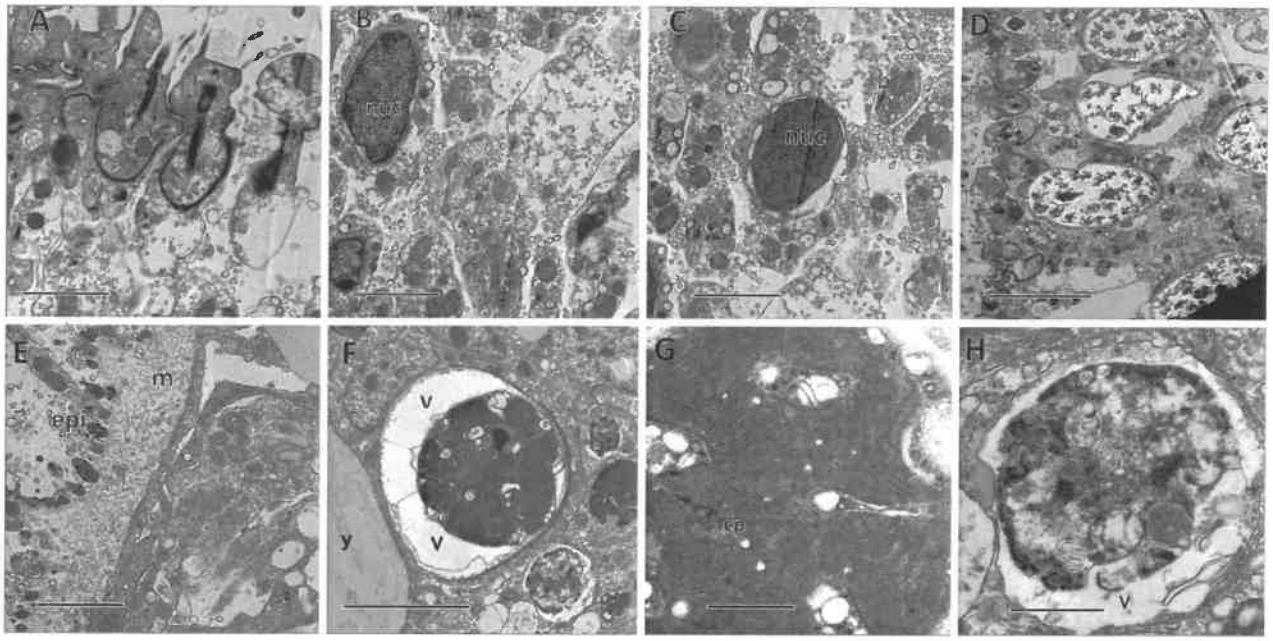

Fig. 5 Transmission electron microscopy of *Stylophora pistillata* planula exposed to 228 parts per billion (μg/L) benzophenone-3 for 8 h in the dark. **a** Surface of the epidermal layer; ciliated cells are present, but undergoing early stages of autophagic cell death. Cells beneath the cilia layer exhibiting late stage autophagic cell death and necrosis. Note scratches in the micrograph; *bar* indicates 2000 nm. **b** Epidermal tissue area between cilia and nematocyst layer showing extensive vacuolization. Early stages of nuclear vacuolization (nuc). Note scratches in the micrograph; *bar* indicates 2000 nm. **c** Epidermal tissue in area exhibiting advanced stages of cell death; nucleus vacuolization (nuc). Note scratches in the micrograph; *bar* indicates 2000 nm. **d** Extensive vacuolization of cells surrounding nematocysts. Note scratches in the micrograph; *bar* indicates 5000 nm. **e** Mesoglea (m), gastrodermal and epidermal tissues. Symbiophagy occurring to zooxanthella (zx) surrounded by extensive vacuolization in neighboring cells; *bar* indicates 2000 nm. **f** Gastrodermal tissue and yolk (y). All cells exhibiting extensive vacuolization (v), especially within the gastrodermal cell surrounding the zooxanthella. Coral cells showing increased level of autophagosome content but no signs of autophagic cell death or necrosis; *bar* indicates 5000 nm. **g** Zooxanthella chloroplast with thylakoid dispersion-pathomorphologies. Chloroplast (cp); *bar* indicates 1000 nm. **h** Zooxanthella exhibiting extensive pyknosis; symbiophagic vacuole (v); *bar* indicates 1000 nm

et al. (2008), viral inclusion bodies were not observed in our electron microscopy examination.

During the initial examination of the planulae using transmission electron microscopy, scratches in the micro-sections under observation were readily apparent (Figs. 5a–c and 6). Scratches to the microsection can arise as a result of hardened particles from the sample that scrape between the diamond blade and micro-sectioned sample (Carson 1997; Crang and Klomparens 1988). This is a common occurrence in biological samples that contain $CaCO_3$ skeleton (coral or vertebrates). These scratches are preventable if the samples are first decalcified before embedding in a resin and sectioned (Crang and Klomparens 1988). Coral planula samples do not normally need to be decalcified, because they should contain no aragonite skeletal matrix. An Alizarin red stain confirmed the presence of a $CaCO_3$ crystal matrix on the surface of the planula (data not shown; Barnes 1972). Decalcifying the fixed coral planulae with EDTA before embedding the sample in resin alleviated the "scratch" artifact and the remaining samples that were processed using a decalcification step were devoid of scratches.

Increasing concentrations of BP-3 induced significantly higher levels of DNA AP lesions in planulae exposed to the light compared to the controls (Fig. 7a, b), as well as planulae exposed to BP-3 in the dark (Fig. 7c, d).

No-Observed-Effect Concentration

Estimating Lowest-observed-effect Concentration (NOECs) for planulae exposed to BP-3 for 8 h was problematic because responses in the control treatment were homogeneous (Shapiro–Wilk; $P < 0.05$); all planulae survived and were not deformed, so analyses defaulted to the less powerful, nonparametric method (Steel 1959). The NOEC for both the proportion of live coral planulae and nondeformed planulae exposed to BP-3 for 8 h in either the light or the dark was 228 ppmillion (mg/L) (Steel Method (Steel 1959), all $Z > 2.32$, $P < 0.0809$; Fig.8a, c). In contrast to the Steel Method, the NOEC for planulae in the light determined by a Kruskal–Wallis One-Way Analysis of Variance on Ranks was 228 μg/L (H Statistic = 21.903; $P \leq 0.001$; Dunnett's Procedure). The NOEC for planulae

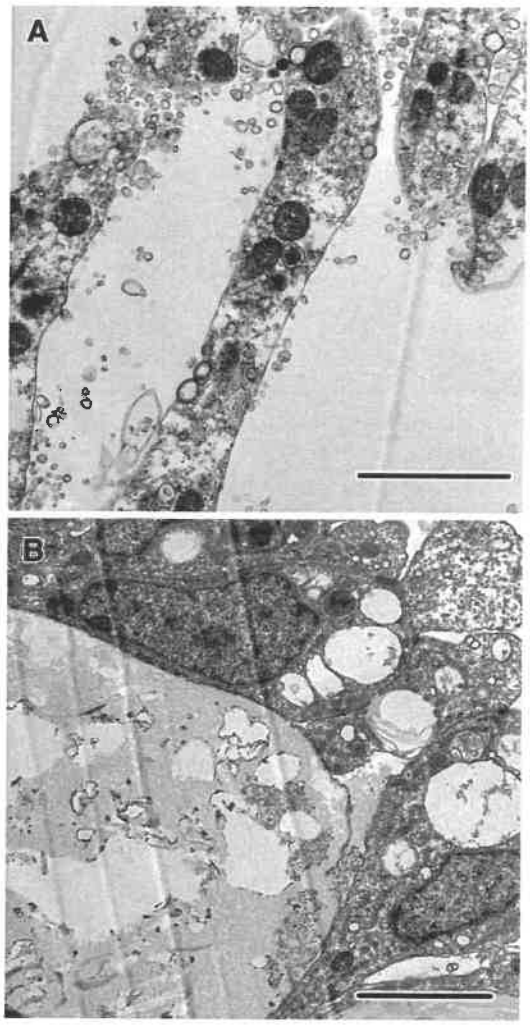

Fig. 6 "Scratch" artifacts in transmission electron microscopy micrographs of *Stylophora pistillata* planula exposed to 288 parts per billion (μg/L) benzophenone-3. When microsectioning planula embedded in a plastic resin without first decalcifying the sample, scratches can manifest on the mounted ultrathin sections. The scratches form as a result of the diamond blade fracturing the aragonite skeleton and pieces of the skeleton adhering to the edge of the diamond blade. As the contaminated blade cuts through the sample block, it scratches the ultrathin sections of the sample. These scratches can be alleviated by cleaning the diamond blade and removing aragonite skeleton in the sample through decalcification before embedding the sample in a resin. **a** Scratches apparent in ultrathin section of epidermal section of a planula; *bar* indicates 2000 nm. **b** Scratches apparent in ultrathin section of gastrodermal section of a planula; *bar* indicated 5000 nm

in the dark determined by a Kruskal–Wallis One-Way Analysis of Variance on Ranks was 228 μg/L (H Statistic = 22.402; $P \leq 0.001$; Dunnett's Procedure).

Estimates for NOECs for planulae exposed to BP-3 for 24 h in light or darkness also were problematic because responses in the control and at all concentrations greater than 22.8 μg/L (in certain cases, ≥2.28 μg/L) were homogeneous (Fig. 8b, d); all planulae survived and were not deformed in the control but died at the higher concentrations (Laskowski 1995). Using the nonparametric Steel Method, we determined the NOEC as 2.28 μg/L for the proportion of coral planulae alive after 24 h of exposure to BP-3 in the light and 22.8 μg/L in the dark (both $Z = 2.48$, $P = 0.0543$). The corresponding NOECs for non-deformed planulae were identical to these values (Fig. 9a, c). In contrast, the NOEC for planulae exposed for 24 h in the light, determined by a Kruskal–Wallis One-Way Analysis of Variance on Ranks, was 228 μg/L (Fig. 9b; H Statistic = 22.084; $P \leq 0.001$; Dunnett's Procedure). The NOEC for planulae exposed for 24 h in darkness, determined by a Kruskal–Wallis One-Way Analysis of Variance on Ranks, was 228 μg/L (Fig. 9d; H Statistic = 22.112; $P \leq 0.001$; Dunnett's Method).

The NOEC for DNA abasic sites in planulae met ANOVA assumptions and was determined as 22.8 μg/L (100 nM; one-way ANOVA $F_{4,15} = 73.1$, $P < 0.0001$, $R^2 = 0.95$; Dunnett's Method for this comparison, $P < 0.0001$) when exposed in the light, and 22.8 μg/L (100 nM) when exposed in the dark (Welch ANOVA $F_{5,7.67} = 142.1$, $P < 0.0001$; Dunnett's Method for this comparison, $P < 0.0001$). The NOEC for mortality of *S. pistillata* calicoblast cells was below the 570 ng/L concentration for cells exposed to the dark for 4 h (Fig. 10a, b). The NOEC for mortality of *S. pistillata* calicoblast cells was 570 ng/L for cells exposed to the light for 4 h (Fig. 10c, d).

LC_{50}, EC_{50}, and EC_{20} Values

Regression models used to estimate median LC_{50} (concentration expected to cause death in 50 % of the population), EC_{20} and median EC_{50} (effective concentrations, which adversely affect 20 and 50 % of the population, respectively) after 8 h of exposure to BP-3 had coefficients of determination (R^2) between (0.91 and 0.97). Using regression models, the median LC_{50} for the proportion of live coral planulae exposed in the light was 3.1 mg/L, whereas for planulae exposed in the dark, the LC_{50} was 5.4 times higher: 16.8 mg/L (Table 1; Supplemental Fig. 1a, c). PROBIT analysis for LC_{50} in the light was 2.876 mg/L (mg/L), whereas LC_{50} in the dark was 12.811 mg/L (Table 1; Supplemental Fig. 2a, c).

Models used to estimate LC_{50} and EC_{50}, of coral planulae after 24 h of exposure to BP-3 continued to explain the substantial variation ($0.86 < R^2 \leq 0.997$). The 24 h-LC_{50} for the proportion of live coral planulae, after exposure in the light, was just 103.8 μg/L (ppbillion) compared with 873.4 μg/L in the dark exposure (Table 1; Supplemental Fig. 1b, d). PROBIT analysis for 24-h LC_{50} in the light was 139 μg/L, whereas LC_{50} in the dark was 799 μg/L (Table 1; Supplemental Fig. 2b, d).

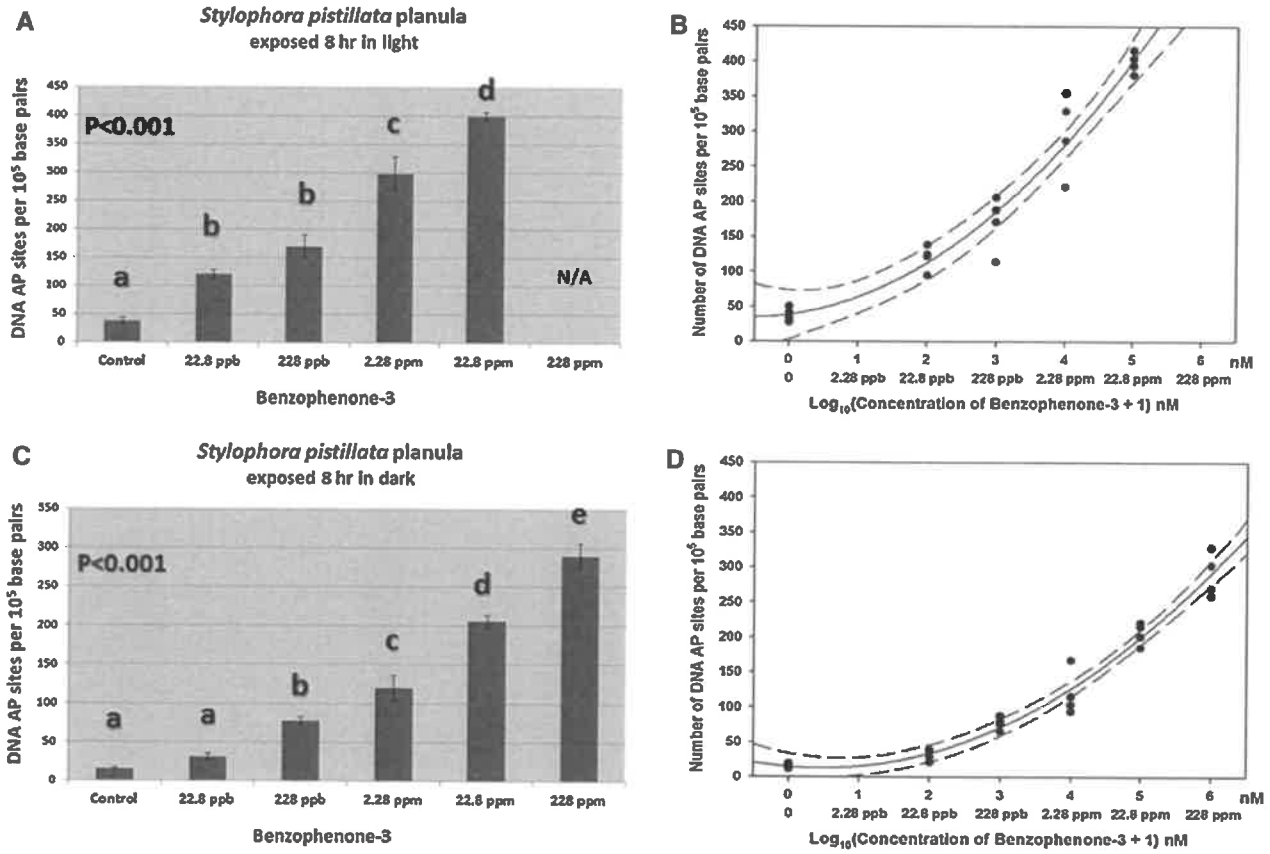

Fig. 7 Number of DNA apyrimidinic lesions in planulae of *Stylophora pistillata* exposed to various concentrations of benzophenone-3 (BP-3). *Bars* show treatment means of four replicates with whiskers representing ±1 standard error of the mean. Treatment means with *different letters* differed significantly at $\alpha = 0.05$, based on Kruskal–Wallis one-way analysis of variance on ranks followed by a Student–Newman–Keuls Method post hoc test. **a** Planulae exposed for 8 h in the light. **b** Log-linear regression between DNA AP lesions of coral planulae of *Stylophora pistillata* exposed to concentrations of BP-3 for 8 h in the light. Quadratic regression line (*solid*) and 95 % confidence intervals (*dashed lines*) are shown. **c** Planulae exposed for 8 h in the dark. **d** Log-linear regression between DNA AP lesions of coral planulae of *Stylophora pistillata* exposed to concentrations of BP-3 for 8 h in the dark

The 8-h EC_{50} for nondeformed planulae exposed to BP-3 in the light and dark were much lower: 107 and 436 µg/L, respectively using regression modeling (Table 1; Supplemental Fig. 3a, c). PROBIT analysis for 8-h EC_{50} in the light was 133 ppbillion (µg/L), whereas EC_{50} in the dark was 737 µg/L (Table 1; Supplemental Fig. 4a, c). PROBIT analysis for 8-h EC_{20} in the light was 6.3 µg/L, whereas EC_{20} in the dark was 15.5 µg/L (Table 1; Supplemental Fig. 4a, c). The 24-h EC_{50} for nondeformed planulae exposed in the light and dark were much lower: 17 ppbillion and 105 µg/L, respectively using regression modeling (Table 1; Supplemental Fig. 3b, d). PROBIT analysis for 24-h EC_{50} in the light was 49 µg/L, whereas LC_{50} in the dark was 137 µg/L (Table 1; Supplemental Fig. 4a, d). PROBIT analysis for 24-h EC_{20} in the light was 6.5 µg/L, whereas EC_{50} in the dark was 10.4 µg/L (Table 1; Supplemental Fig. 4b, d).

The number of DNA abasic sites increased approximately tenfold across the BP-3 concentration gradient in the light, but nearly 20-fold in the dark (Fig. 7b, d). Similarly, the percentage of dead coral cells increased dramatically with increasing concentrations of BP-3, but the LC_{50} was much lower in the light at 39 µg/L than in the dark at 842 µg/L. PROBIT analysis for 4-h LC_{50} coral cells in the light was 42 ppbillion, whereas LC_{50} in the dark it was 679 µg/L (Table 2; Supplemental Fig. 5a, b).

Species Sensitivity Distribution Using Coral Cell Toxicity Assay

To provide a perspective of the differences in sensitivities of various species of Indo-Pacific and Caribbean coral reefs, the LC_{50}s and LC_{20}s with their corresponding upper

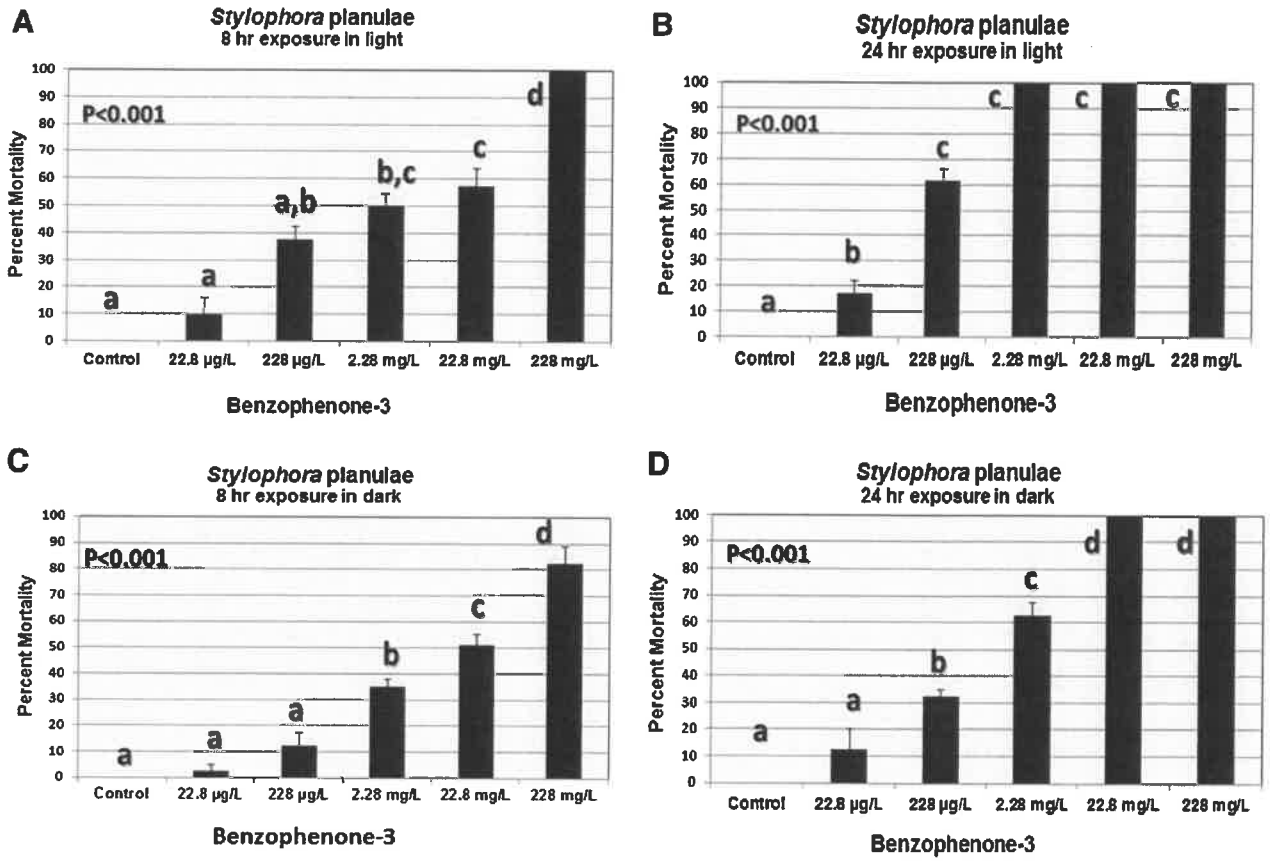

Fig. 8 Percent mortality of planula of *Stylophora pistillata* exposed to various concentrations of benzophenone-3. *Bars* show treatment means with whiskers representing ±1 standard error of the mean. Treatment means with *different letters* differed significantly at $\alpha = 0.05$, based on Kruskal–Wallis one-way analysis of variance on ranks followed by a Student–Newman–Keuls Method post hoc test. **a** Planulae exposed for 8 h in the light. **b** Planulae exposed for 8 h in the light and then 16 h of darkness. **c** Planulae exposed for 8 h in the dark. **d** Planulae exposed for 24 h in the dark

and lower 95 % confidence intervals for the two Indo-Pacific and five Caribbean species are provided in Table 1.

Correction Factor Between Mortality of Coral Planulae and Coral Cells

Coral cells were much more sensitive than coral planulae across a wide range of BP-3 concentrations, which makes cell mortality a potential indicator of reproductive and recruitment failures. To estimate the correction factor needed to translate coral cell mortality into potential mortality of coral planulae, one option is the use of a quadratic regression model to estimate these relationships: In the light ($F_{2,21} = 43.8$, $P < 0.0001$, $R^2 = 0.81$) % mortality of planulae = 2.26 − 0.28 (% mortality of cells) + 0.0107 (% mortality of cells)^2 In the dark ($F_{2,21} = 84.5$, $P < 0.0001$, $R^2 = 0.89$) % mortality of planulae = 0.86 − 0.0007 (% mortality of cells) + 0.0078 (% mortality of cells)^2

Environmental Chemistry Analysis

The purpose of the chemical analysis was to conduct a cursory survey of BP-3 concentrations on coral reefs. Seawater samples were collected from bays in St. John Island, U.S. Virgin Islands: Caneel Bay, Hawksnest Bay, and Trunk Bay in April 2007 (Fig. 11a, b). Caneel Beach is managed by the resort, Caneel Bay. Samples were collected at approximately 16:30 h near the dive platform that adjoins the Caneel Beach and along a large coral community that spans from the edge of Caneel Beach to the edge of Honeymoon Beach. There were 17 swimmers in Caneel Bay in the 48-h period before sampling. Swimmers were monitored from the shore of the resort from dawn to dusk. No benzophenones could be detected in either of the samples collected in Caneel Bay.

Hawksnest Bay is a densely visited beach within the U.S. National Park system on St. John Island. In general, more than 1000 visitors per day can enter into this bay. On the day of sampling, more than 230 people entered the

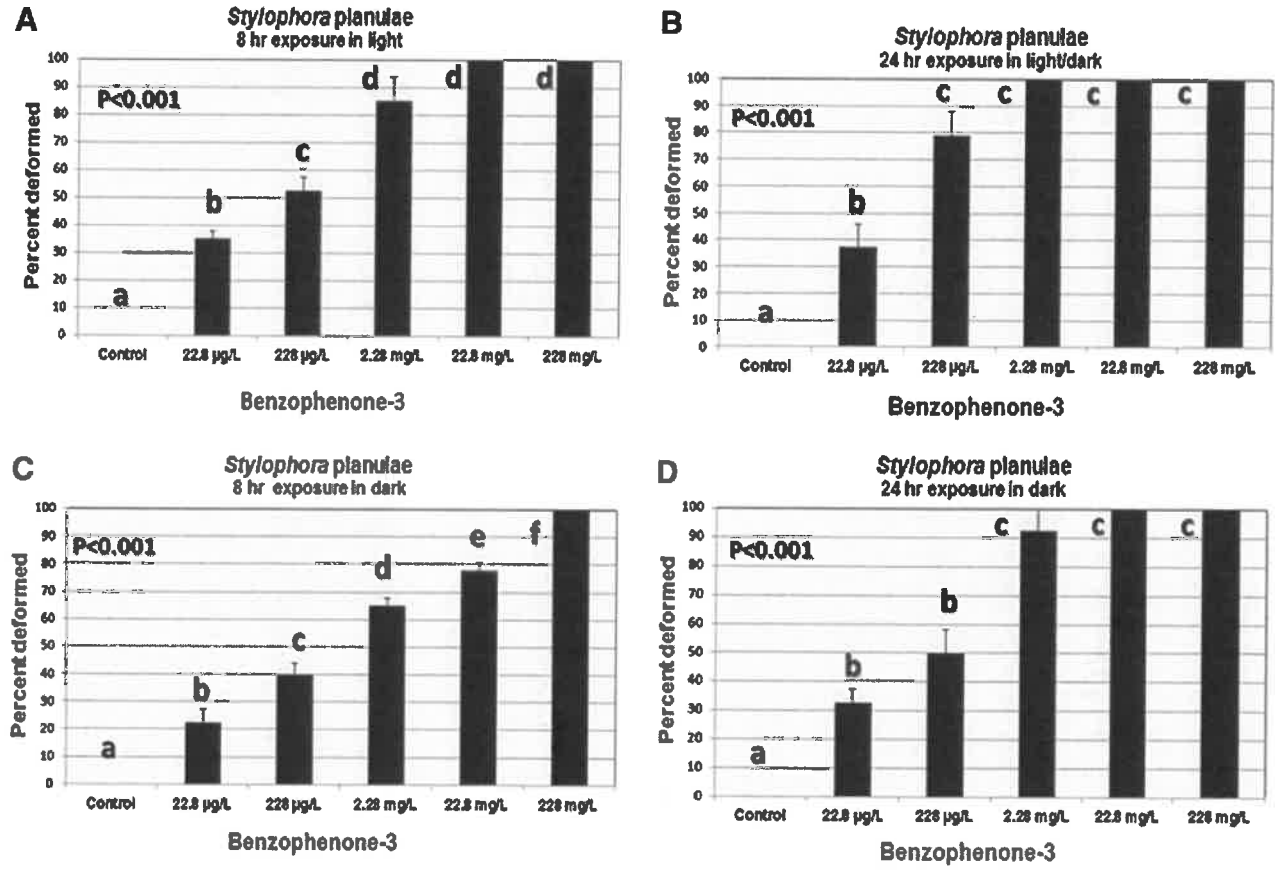

Fig. 9 Percentage of deformed planulae of *Stylophora pistillata* exposed to various concentrations of benzophenone-3. *Bars* show treatment means with whiskers representing ±1 standard error of the mean. Treatment means with *different letters* differed significantly at $\alpha = 0.05$, based on Kruskal–Wallis one-way analysis of variance on ranks followed by a Student–Newman–Keuls Method post hoc test. **a** Planulae exposed for 8 h in the light. **b** Planulae exposed for 8 h in the light, then 16 h of darkness. **c** Planulae exposed for 8 h in the dark. **d** Planulae exposed for 24 h in the dark

water and swam within 20 m of the three large *Acropora palmata* spurs (coral reefs) indicated in Fig. 11c; the majority swam in the sandy grooves that lie between the coral-reef spurs. These spurs are very shallow (1–3 m deep), with live coral often protruding above the surface of the water during low tide. The concentration of BP-3 in the western groove was 75 ppbillion (µg/L), whereas the larger, eastern groove had a BP-3 level of 95 ppbillion (µg/L). Samples were collected between 17:00 and 17:40 h.

Trunk Bay is an iconic landscape and a highly managed natural resource area. Before 2009, there could be more than 3000 visitors on the beach and in the water at Trunk Bay. After 2009, National Park Service policy reduced the number to 2000 visitors per day (personal communication, Rafe Boulon, retired, USVI NP Chief, Resource Management). A coral community surrounds the island in Trunk Bay, as well as an abundance of gorgonians to the west of the island, and there was once a very extensive stand of *A. palmata* corals to the east of the island. At a site near the edge of the Trunk Island coral community, BP-3 levels were 1.395 ppmillion (mg/L) (Fig. 11d). A sampling site 93 m east of the first sampling site contained 580 ppbillion (µg/L) BP-3 (Fig. 11d). Samples were collected at 11:00–11:24 h with more than ~180 swimmers in the water and ~130 sunbathers on the beach within 100 m of the two sampling sites.

Seawater samples were collected at five sites in Maunalua Bay, Oahu Island, Hawai'i on May 30, 2011 between 11:00 and 15:00 h (Fig. 12a, b). ASW samples were collected in public swimming areas in waters that were 1.3 m in depth and 35 cm from the surface of the water. Sites 1–4 had detectable levels of BP-3 (>100 pptrillion; ng/L) but were below the quantitative range of measurement (5 ppbillion (µg/L); Fig. 12b). Site 5 contained measurable levels of BP-3—19.2 ppbillion (µg/L) (Supplemental Fig. 6).

Samples were collected at two sites on June 3, 2011, along the northwest coast of Maui Island, Hawai'i (Fig. 12c). Kapalua Bay is a protected cove and has a

public beach that can often see >500 swimmers/day in the peak tourism season (personal communication, Kapalua Dive Co.; Fig. 12d). A seawater sample was collected 40 m from shore near the center of the bay, immediately above remnants of a coral reef at 09:30 h. The Kapalua sample had detectable levels of BP-3 but was below the quantitative range of measurement (5 ppbillion, 5 μg/L). From 06:30 to 09:30 h on the day of sampling, 14 swimmers had entered Kapaula waters. A seawater sample also was collected at Kahekili Beach Park, Maui Island, Hawai'i (Fig. 12e). Kahekili Beach is a public beach that also serves visitors from a number of nearby hotels and resorts. The sample was collected 30 m from shore, immediately above a coral reef. Unlike Kapalua, Kahekili is an exposed shoreline not protected within a bay, and retention time of contaminants is thought to be minimal because of the prevailing currents. The Kahekili sample had detectable levels of BP-3 but was below the quantitative range of measurement (5 ppbillion). Kahekili is a heavily visited beach and had 71 swimmers within 200 m of the sampling site at the time of sampling (11:45 h).

Table 1 Regression and PROBIT determination of LC_{50} for planulae mortality when exposed to BP-3 in the light and dark, and the EC_{50} for planulae deformity when exposed to BP-3 in the light and the dark

Planulae mortality	LC_{50}
Regression to estimate LC_{50} 8-h light	3.1 mg/L
PROBIT to estimate LC_{50} 8-h light	2.9 mg/L
Regression to estimate LC_{50} 8-h dark	16.8 mg/L
PROBIT to estimate LC_{50} 8-h dark	12.8 mg/L
Regression to estimate LC_{50} 24-h light	103.8 μg/L
PROBIT to estimate LC_{50} 24-h light	1.39 μg/L
Regression to estimate LC_{50} 24-h dark	873.4 μg/L
PROBIT to estimate LC_{50} 24-h dark	799 μg/L
Planulae deformation	**EC_{50}**
Regression to estimate EC_{50} 8-h light	107 mg/L
PROBIT to estimate EC_{50} 8-h light	133 mg/L
Regression to estimate EC_{50} 8-h dark	436 mg/L
PROBIT to estimate EC_{50} 8-h dark	737 mg/L
Regression to estimate EC_{50} 24-h light	17 μg/L
PROBIT to estimate EC_{50} 24-h light	49 μg/L
Regression to estimate EC_{50} 24-h dark	105 μg/L
PROBIT to estimate EC_{50} 24-h dark	137 μg/L
Planulae deformation	**EC_{20} (μg/L)**
PROBIT to estimate EC_{20} 8-h light	6.3
PROBIT to estimate EC_{20} 8-h dark	15.5
PROBIT to estimate EC_{20} 24-h light	6.5
PROBIT to estimate EC_{20} 24-h dark	10.4

PROBIT determination of EC_{20} for planulae deformity when exposed to BP-3 in the light and the dark

Discussion

Toxicopathology

Benzophenone-3 is a phototoxicant and induces different toxicities depending on whether the planulae are exposed to the chemical in light or in darkness. Corals will usually release brooded planulae at night or spawn gametes at night (Gleason and Hofmann 2011). Planulae of broadcasting species (those that spawn eggs and sperm that are fertilized in the water column) are positively buoyant and planktonic, residing at or near the surface of the ocean for 2–4 days before they are able to settle (Fadlallah 1983; Shlesinger and Loya 1985; Harii et al. 2007; Baird et al. 2009). Light levels on a clear sunny day in tropic latitudes can be as high as or higher than 2000 μmol/m^2/s of photosynthetically active radiation—five times more than what the corals experienced in this study, suggesting that actual environmental conditions may aggravate the phototoxicity. Whether the BP-3 pollution comes from swimmers, or from point and nonpoint wastewater sources, planulae will be at

Table 2 Differences in sensitivities of various species of Indo-Pacific and Caribbean coral reefs, the LC_{50}s and LC_{20}s of calicoblast cells exposed in vitro to benzophenone-3 with their corresponding upper and lower 95 % confidence intervals for the two Indo-Pacific and five Caribbean species. (μg/L) = to parts per billion. (ng/L) = parts per trillion

Coral species	LC_{50} (μg/L)	95 % CI	LC_{20}	95 % CI
Indo-Pacific species				
Stylophora pistillata (light)	42	28; 60	2 μg/L	1.14; 3.61
Stylophora pistillata (dark)	671	447; 984	14 μg/L	7; 26
Pocillopora damicornis	8	4.96; 12.15	62 ng/L	24; 136
Caribbean-Atlantic species				
Acropora cervicornis	9	5.4; 14.5	63 ng/L	22; 150
Montastrea annularis	74	40; 126	562 ng/L	166; 1459
Montastrea cavernosa	52	36; 72	502 ng/L	247; 921
Porites astreoides	340	208; 534	8 μg/L	3; 16
Porites divaricata	36	21; 57	175 ng/L	60; 420

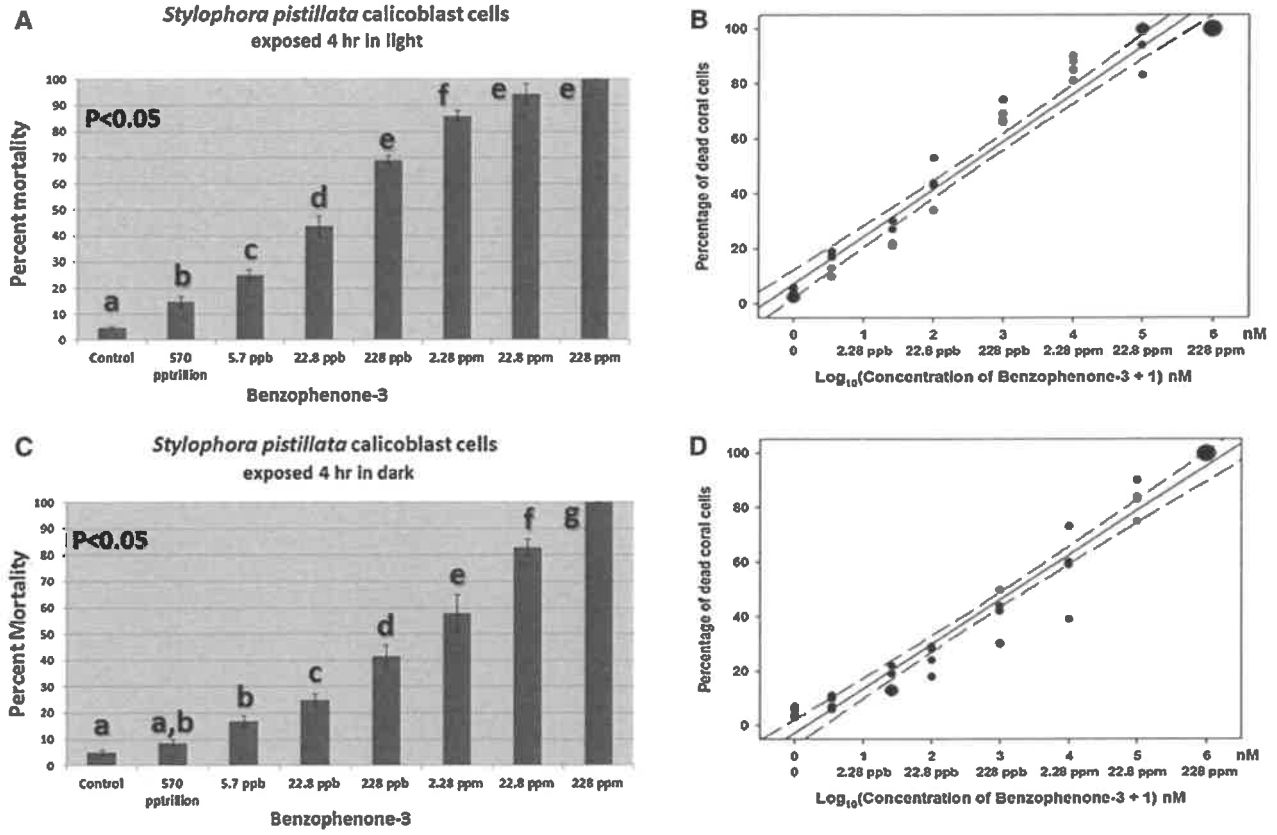

Fig. 10 Percentage mortality of calicoblast cells of *Stylophora pistillata* exposed to various concentrations of benzophenone-3. *Bars* show treatment means ($n = 4$) with whiskers representing ± 1 standard error of the mean. Treatment means with *different letters* differed significantly at $\alpha = 0.05$, based on one-way analysis of variance followed by a Tukey's Honestly Significant Difference Test. **a** Calicoblast cells exposed for 4 h in the light. **b** Log-linear regression between coral cell mortality and concentrations of BP-3 for 4 h in the light. Quadratic regression line (*solid*) and 95 % confidence intervals (*dashed lines*) are shown. *Larger symbols* represent multiple coincident data points, with symbol area proportional to the number of replicates with the same value. **c** Calicoblast cells exposed for 4 h in the dark. **d** Log-linear regression between coral cell mortality and concentration of BP-3 for 4 h in the dark

risk from both forms of toxicities (Brooks et al. 2009; Futch et al. 2010; Pitarch et al. 2010).

As with our previous paper examining benzophenone-2 (Downs et al. 2014), the data in this paper are consistent with the observation by Danovaro et al. (2008) that "sunscreens compounds" cause coral bleaching. In the light, BP-3 caused injury directly to the zooxanthellae, independent of any host-regulated degradation mechanism. Based on the pathomorphology of the thylakoids within the chloroplasts, the most probable interpretation is that BP-3 induces photo-oxidative stress to the molecular structures that form the thylakoid membranes (Downs et al. 2013). In darkness, bleaching resulted from the symbiophagy of the symbiotic zooxanthellae; a process whereby the coral gastrodermal cell "digests" the zooxanthella (Downs et al. 2009). Nesa et al. (2012) demonstrated that following exposure to light, the algal symbionts of corals increased the DNA damage to coral cells in coral planulae. Consistent with the Oxidative Theory of Coral Bleaching (Downs et al. 2002), Nesa et al. hypothesized that the sources of this damage was the production of oxygen radicals. If this is the case, then darkness-associated, BP-3-induced bleaching may reduce the exacerbated morbidity experienced by "bleached" planulae that would occur during the periods of daylight. Regardless of the toxicological mechanism, managing exposure of corals to BP-3 corals will be critical for managing coral reef resilience in the face of climate-change pressures associated with coral bleaching (West and Salm 2003).

Autophagy was the dominant cellular response to BP-3 exposure (Figs. 4a–f, 5b–d; Yla-Antilla et al. 2009). Microautophagosomes were abundant in all cell types and larger vacuolated bodies of specific organelles were readily observed. None of the nuclei in any coral cell-types exhibited any of the classic signs of apoptosis, such as pyknosis or karyorrhexis of the nucleus (Krysko et al.

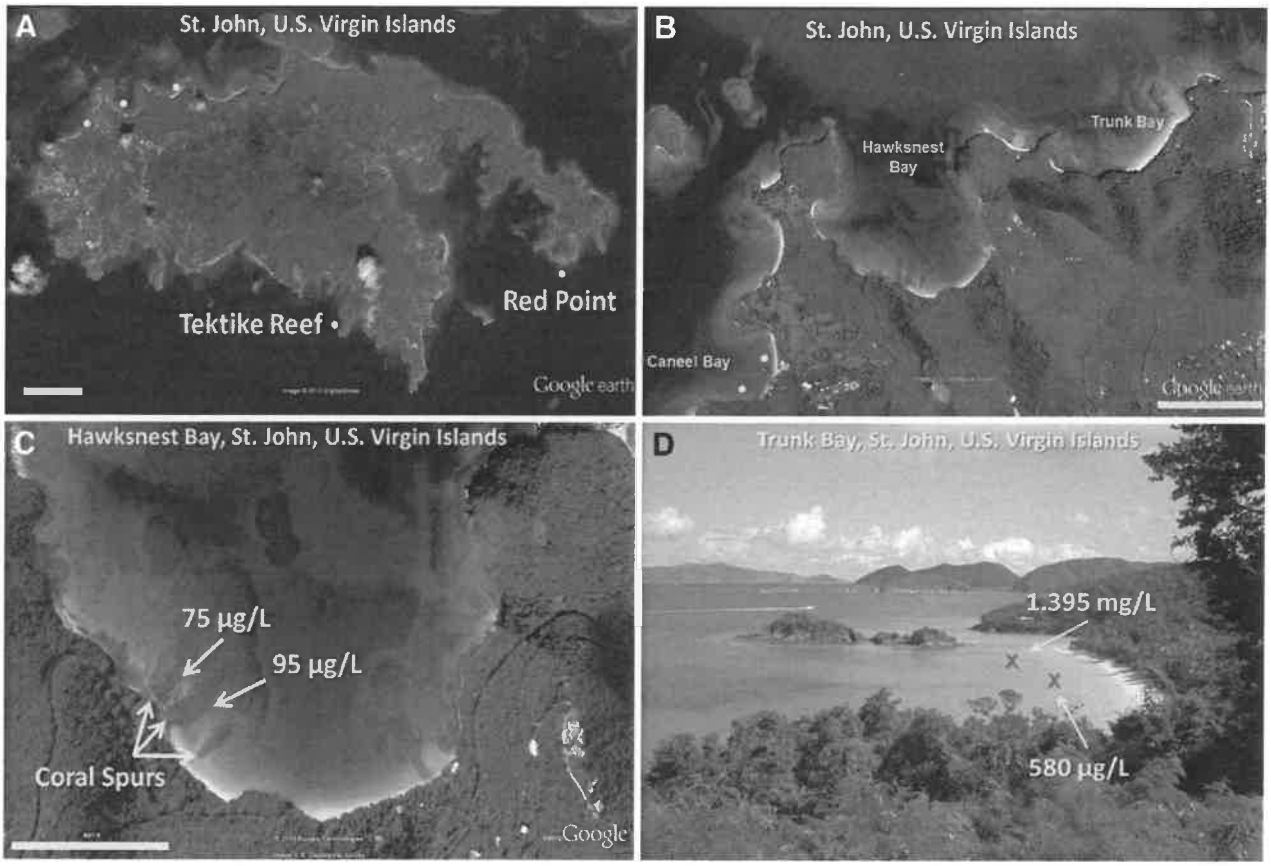

Fig. 11 Seawater analysis of benzophenone-3 (BP-3) in coral reef areas in St. John Island, U.S. Virgin Islands. **a** Aerial view of St. John indicating the five sampling sites, indicated by a *yellow dot*. No benzophenones were detected in samples from *Red Point* or at Tektite Reef. All samples were taken between 12:00 and 14:00 h. *Scale bar* is 1.5 km. **b** Aerial view of the three northwestern sites within St. John National Park: Trunk Bay, Hawksnest Bay, and Caneel Bay. The two sampling sites at Caneel Bay are indicated by *yellow dots*. No benzophenones were detected in samples from Caneel Bay. *Scale bar* is 500 m. **c** Aerial view of the two sampling sites in Hawksnest Bay, St. John Island. *Yellow arrows* indicate three coral reef spurs that are dominated by the U.S. Threatened Species, *Acropora palmata*. *Yellow arrows* pointing at *red dots* indicate the sample site. *Values* indicate the concentration of BP-3 in the water column. *Scale bar* is 245 m. **d** Elevated view of Trunk Bay, St. John Island. *Yellow arrows* pointing to *red* "X" indicate the sample site. The *values* indicate the concentration of BP-3 in the water column at those two sites

2008). The most fascinating aspect of these autophagic events were the delamination of the nuclear bilayer membrane (Figs. 4b, c, 5b, c), a classic hallmark of autophagic cell death and further evidence arguing against apoptosis as a regulated mechanism of cnidarian cell death (Tasdemir et al. 2008; Yla-Antilla et al. 2009; Klionsky et al. 2012). In both the light and the dark, there was a gradation of vitiated cells beginning at the surface of the epidermis to "non-morbid" cells in the gastrodermis that surrounded the yolk. In Figs. 4a and 5a, the cells are severely degraded; it is difficult to distinguish any mechanism of cell death, and the cells could easily be labeled as necrotic. Going 20,000 nm into the planula from the surface, cells exhibited the hallmarks of autophagic cell death. This tissue transect of the gradation of cell death is evidence for a model of cell death, first demonstrated in *C. elegans*, that requires autophagic degradation of cells for the manifestation of necrosis (Samara et al. 2008; Eskelinin et al. 2011).

BP-3 is a genotoxicant to corals, and its genotoxicity is exacerbated by light. Based on the current literature, this was not unexpected, but our data do underscore the threat that BP-3 may pose to not only corals but also to other coral-reef organisms (Hanson et al. 2006; Cuquerella et al. 2012). DNA AP lesions can be produced in response to oxidative interaction or alkylation events (Fortini et al. 1996; Drablos et al. 2004). Accumulation of DNA damage in the larval state has implications not only for the success of coral recruitment and juvenile survival, but also for reproductive effort and success as a whole (Anderson and Wild 1994; Depledge and Billinghurst 1999). Surviving planulae exposed to BP-3 may settle, metamorphose, and

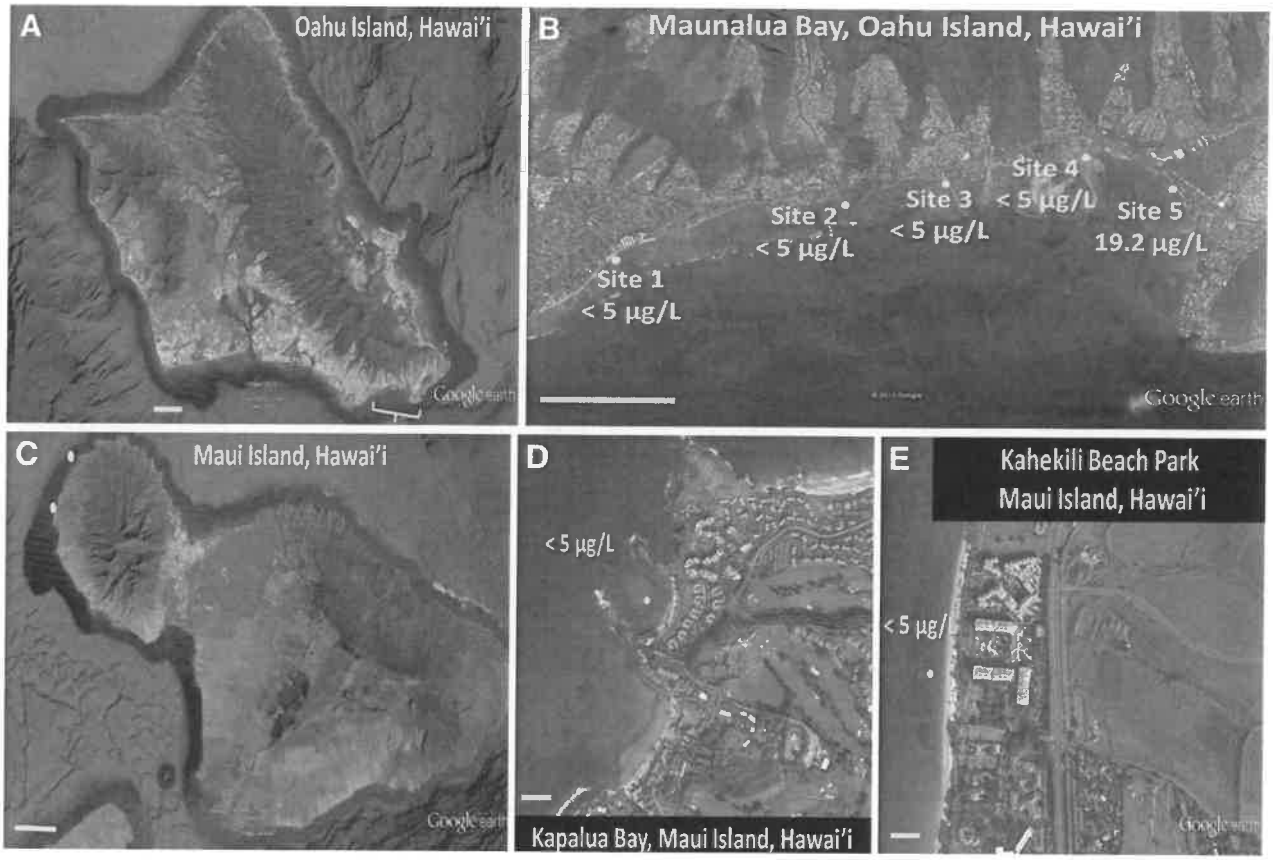

Fig. 12 Seawater analysis of benzophenone-3 (BP-3) in coral reef areas in Oahu and Maui islands, Hawai'i. *Yellow dots* indicate the sampling location in the panels. **a** Aerial view of Oahu indicating the five sampling sites. *Scale bar* is 5 km. **b** Aerial view of the five sampling site along the coast of Maunalua Bay, Oahu. Sites 1–4 had levels of BP-3 that were detectable, but below the quantitative range. *Scale bar* is 1.5 km. **c** Aerial view of the two sampling sites in Maui, Hawai'i. *Scale bar* is 6 km. **d** Elevated view of Kapalua Bay, Maui. *Scale bar* is 100 m. **e** Elevated view of Kahekili Beach, Maui. *Scale bar* is 100 m

develop into colonial adults, but they may be unfit to meet the challenges of other stressor events, such as increased sea-surface temperatures. Cnidarians are rather unusual in the animal kingdom in that the germline is not sequestered away from the somatic tissue in early stages of development; the somatic tissue gives rise directly to the germline during seasonal reproductive cycles. Damage to the genomic integrity of coral planulae therefore may have far-reaching and adverse impacts on the fitness of both the gametes in adults.

The ossification of the planulae from exposure to BP-3 is one of the strangest cases of developmental endocrine disruption to wildlife, although skeletal endocrine disruption in vertebrates is only now being recognized (Colburn et al. 1993; Depledge and Billinghurst 1999; Golub et al. 2004; Lind et al. 2004; Doherty et al. 2004; Agas et al. 2013). In mammals, estrogen and estrogenic compounds may influence different estrogen and thyroid hormone receptors, which affect bone growth and composition (Rickard et al. 1999; Lindberg et al. 2001; Golub et al. 2004). In classic vertebrate physiology, estrogen plays a complex role in ossification and skeletal maintenance, affecting both bone anabolism and catabolism (Simmons 1966; Väänänen and Härkönen 1996). In vertebrates, exposure to high levels of estrogen can result in skeletal hyperossification (Pfeiffer et al. 1940; Rickard et al. 1999). For "classic" endocrine disruptors, such as tributyltin and dioxin, ossification is inhibited, not induced (Birnbaum 1995; Jamsa et al. 2001; Tsukamoto et al. 2004; Finnila et al. 2010; Agas et al. 2013). Osteo-endocrine disruption is both complex and complicated; different compounds affect different cell types within the skeletal tissue differently (Hagiwara et al. 2008a, b; Agas et al. 2013). Benzophenones as endocrine disruptors are no exception; BP-3 and BP-2 showed contradictory effects on estrogen and aryl hydrocarbon receptors, and both compounds induced "…a kind of endocrine disruption that is not assessed by 'classical' estrogenic markers" (Schlecht et al. 2004; Seidlová-Wuttke et al. 2004; Ziolkowaska et al. 2006).

The ossification-induced opacity of the epidermal tissue layer of planulae was readily observed at the three highest

concentrations of BP-3 exposure but was not visually obvious at the lower concentrations, although we know from the electron microscopy sample processing that ossification was present to a lesser extent in the lower BP-3 exposures. Many endocrine disruptors do not exhibit a "classic" monotonic exposure–response curve, but instead exhibit nonmonotonic behaviors (vom Saal et al. 1995; Conolly and Lutz 2004; http://epa.gov/ncct/edr/non-monotonic.html). Ossification of planulae can be assayed by a variety of methods, including alizarin staining and calcein fluorescence. This study was not designed to be an exhaustive characterization of exposure–response behavior (i.e., regulatory toxicology); hence lower BP-3 exposure concentrations were not attempted. More comprehensive studies that examine the ossification response of both acute and chronic exposure of BP-3 in the lower pptrillion and ppquadrillion need to be conducted to determine accurately this endocrine behavioral response.

Ecotoxicology and Species Sensitivity

To conduct a relevant and accurate ecological risk or threat assessment, it is imperative that the species chosen reflects the structure of the specific coral-reef ecosystem being affected (Suter 2007). *Stylophora pistillata* used in this study, is indigenous to specific regions in the Indo-Pacific basins, and hence may not be a valid representative for coral-reef communities in Hawaii or the Atlantic/Caribbean basins. The use of coral planulae in research studies is a relatively difficult resource to obtain. It requires access to healthy coral colonies that are reproductively viable, spawning in specific dates and specific moon phases, and in addition, obtaining the necessary collection and transport permits. We therefore applied an in vitro primary cell toxicity methodology using a specific coral cell type that has been proposed as a surrogate for either planula or colonial polyp studies (Downs 2010). Comparison of LC_{50}s of coral cells in the light (42 ppbillion; μg/L) and coral planula in the light for 8 and 24 h [2.876 ppmillion (mg/L) and 139 ppbillion (μg/L), respectively] exhibits a similar response. The increased sensitivity of in vitro cell models versus whole organism models is a common phenomenon and accepted principle (Blaauboer 2008; Gura 2008). Diffusion of BP-3 across the epidermal boundary layer and reaching concentrations that are toxic in the interior of the planula (e.g., gastroderm) versus direct exposure by cultured cells could likely be the major factor influencing the variation in LC_{50} rate. Although there are obvious caveats to using in vitro models, this may be the only way to conduct ecotoxicological research and ecological risk assessments on coral species that are currently endangered with extinction, such as the species on the IUCN's Red List or species proposed/listed for protection under the U.S. Endangered Species Act.

When an environmental stressor impacts a community of organisms, different species may respond (tolerate) dissimilarly to one another; some species may tolerate the stressor at a particular level, whereas other species may succumb (Johnston and Roberts 2009; Maloney et al. 2011). This species sensitivity distribution is a crucial concept for ecological risk assessments and a predictor of the species composition of a community (community phase-shift) in reacting to a pollution stressor, as well as defining the probability of success for community/ecological restoration (Posthuma et al. 2002; van Woesik et al. 2012). This concept readily applies to corals and coral reefs. Coral bleaching in response to heat stress or freshwater input is an excellent example of this community behavior; some species have high tolerance to stress-induced bleaching, whereas others are highly susceptible, resulting in species-specific extinctions in localized areas (Goreau 1990; Loya et al. 2001; Jimenez and Cortes 2003). Species sensitivity distribution in response to pollutants in corals is also well documented, including synergisms between pollutants and heat stress (Loya 1975; Brown 2000; Fabricius 2005). For the Caribbean, the species sensitivity to BP-3 toxicity is consistent with the model for coral tolerance to general stress as set forth by Gates and Edmunds (1999): corals with slower growth rates, such as massive or boulder coral species, are inherently more tolerant than coral species with higher growth rates (e.g., branched species such as *A. cervicornis* and *P. divaricata*). In fringing reefs that have been impacted by anthropogenic stressors, especially fringing reefs near tourist beaches, *Acropora* species are the first to experience localized extinction. Species that tentatively endure a decade or longer of sustained stress, but are intermediate in their persistence, are the large boulder corals found in the genus *Montastrea* (synomym *Orbicella*). Coral cell toxicity data indicated that *P. astreoides* was at least 4.5× more tolerant to BP-3 toxicity than the second more tolerant coral species and at least 38× more tolerant than the most sensitive species. This is consistent with observations that *P. astreoides* is usually the last to become extinct in a polluted-impacted locality and one of the first to recruit once water quality parameters reach a minimum level of habitability (Peters 1984; Lirman et al. 2003; Alcolado-Prieto et al. 2012). From a management perspective, these data can be used to predict the changes in coral-reef community structure when challenged with BP-3, regarding which species will become extinct, as well as the species that will persist in areas that are adjacent to tourist beaches, popular mooring sites, or near sewage discharges. These data also can be integrated directly into reef resilience management

plans against climate change, acting as both a measurable endpoint for management effectiveness and as a target (concentration of BP-3 in seawater on a reef) for establishing action values for reef management.

Management of BP-3 Pollution for Coral Reef Conservation and Restoration

What do these pathological toxicities induced by BP-3 mean demographically and ecologically for corals and coral reefs? Trunk Bay in St. John Island, the U.S Virgin Islands, may represent an example of this effect. Ecologically, this area has been severely degraded in the past 25 years, despite the limited input from human activities in the watershed and from marine sources. The most obvious input is recreational swimming at Trunk (Downs et al. 2011). During our monitoring of this site from 2005 to 2010, settlement of planulae and recruitment/survival of juvenile coral was almost 0 %. Established coral colonies in this area were assayed for regeneration of tissue over experimentally induced lesions (laceration-regeneration assay, a single diagnostic test for the general health of a coral; Fisher et al. 2007); not a single colony exhibited any regeneration of any of the lesions during the 5-year investigation (Downs et al. 2011). This was in contrast with Caneel Bay, which had undetectable levels of BP-3 resulting from a much lower density/rate of swimmers and has a flourishing coral community on its southern bank with an abundance of recruitment. These demographic-level pathologies are consistent with the pathologies that manifest from BP-3 exposure. The pathologies exhibited at this site can be seen at other coral reef swimming areas the world over: Eilat, Israel (degraded with an abundance of sunscreen lotion users) versus Aqaba, Jordan (thriving coral reefs with swimmers that do not use sunscreen lotion; Fuad Al-horani, personal communication), Honolua Bay in Maui, Hawaii, Hanauma Bay Beach in Oahu, Hawaii, Seven Mile Beach in Grand Cayman, Bathway Beach in Grenada, Playa Langosta, and Playa Tortugas Beaches in Cancun, Mexico. At Okinawa, Tashiro and Kameda (2013) demonstrated that BP-3 contamination from beaches can travel over 0.6 km in distance from the pollution source. The threat of BP-3 to corals and coral reefs from swimmers and point and non-point sources of waste-water could thus be far more extensive than just a few meters surrounding the swimming area.

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Compliance with Ethical Standards

Conflict of Interest The authors can identify no potential conflicts of interest, neither financial nor ethically, involved in the writing or publication of this manuscript.

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REVIEW ARTICLE

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Dermatological and environmental toxicological impact of the sunscreen ingredient oxybenzone/benzophenone-3

Joseph C DiNardo MS[1] | Craig A Downs PhD[2]

[1]Research Scientist Emeritus, Vesuvius, VA, USA

[2]Haereticus Environmental Laboratory, Clifford, VA, USA

Correspondence
Joseph C DiNardo, Research Scientist Emeritus, Vesuvius, VA, USA.
Email: jmjdinardo@aol.com

Summary

Oxybenzone (Benzophenone-3) is an emerging human and environmental contaminant used in sunscreens and personal care products to help minimize the damaging effects of ultraviolet radiation. The Center for Disease Control fourth national report on human exposure to environmental chemicals demonstrated that approximately 97% of the people tested have oxybenzone present in their urine, and independent scientists have reported various concentrations in waterways and fish worldwide. Oxybenzone can also react with chlorine, producing hazardous by-products that can concentrate in swimming pools and wastewater treatment plants. Moreover, adverse reactions could very well be increased by the closed loop of ingesting fish contaminated with oxybenzone and/or washing the ingredient off our bodies and having it return in drinking water as treatment plants do not effectively remove the chemical as part of their processing protocols. In humans, oxybenzone has been reported to produce contact and photocontact allergy reactions, implemented as a possible endocrine disruptor and has been linked to Hirschsprung's disease. Environmentally, oxybenzone has been shown to produce a variety of toxic reactions in coral and fish ranging from reef bleaching to mortality. Lastly, with the rise in skin cancer rates and the availability of more effective sunscreen actives such as micronized zinc oxide and titanium dioxide, serious doubts about the relative prevention benefit of personal care products containing oxybenzone must be raised and compared with the potential negative health and environmental effects caused by the accumulation of this and other chemicals in the ecosystem.

KEYWORDS
contact dermatitis, environmental contaminant, toxicity

1 | INTRODUCTION

Consumer awareness about human health and environmental concerns associated with various ingredients used in personal care products is increasing markedly. Several state and Federal laws banning the use of polyethylene microbeads in cleansing scrubs, tooth pastes, and other consumer products were instituted in 2016 as a result of their presence in numerous fish species found in the food supply and the associated potential adverse health effects to humans.[1] In 2017, several bills have been introduced in the Hawaiian legislature that are designed to ban the use of oxybenzone in any consumer product—particularly if the intended use is near beaches—or, at a minimum, requiring a warning label stating that the chemical is harmful to coral and the aquatic environment. Oxybenzone is an aromatic hydrocarbon that acts as an ultraviolet (UV) light filter in sunscreen formulations. As would be expected, there has been significant debate regarding these proposed actions, with environmentalist calling for a ban on the chemical, industry voices questioning the scientific validity of the negative human/environmental toxicity data based on limited safety data conducted 20 to 40 years ago, and the

medical profession expressing concerns related to increasing the rate of skin cancer should UV blockers, like oxybenzone, be removed from sunscreen formulations. The present review examines the scientific evidence related to oxybenzone and posits that alternative formulation strategies using micronized zinc oxide and/or titanium dioxide are available which avoid the toxic effects. It is hoped that this examination will be useful to the dermatology community as it considers how to best respond to patient questions related to human health and environmental concerns associated with the use of oxybenzone.

2 | GENERAL INFORMATION

Common Name used on Drug Labels (Active Ingredient): Oxybenzone.

Common Name used on Non-Drug Labels (INCI Name): Benzophenone-3.

Common Technical/Chemical Name: 2-Hydroxy-4-Methoxyphenyl Phenylmethanone.

Common Trade Names: Eusolex 4360 and Escalol 567.

Chemical Abstract Service (CAS) Number: 131-57-7.

Molecular Weight (MW): 228.26 Daltons (g/mol).

3 | USES

Oxybenzone is commonly used as a short-wave (290 to 320 nm) ultraviolet light (UVB) and mainly short-wave UVA light (320 to 340 nm) absorber at concentrations up to 6% in sunscreen preparations and up to 0.5% in personal care products as a photo-stabilizer minimizing color and odor changes. It has been reported to be used in over 2000 personal care formulations spanning numerous product categories from skin and hair care to color cosmetics and fragrances. Additionally, it is used in plastics as an ultraviolet light absorber and stabilizer. In 1990, oxybenzone was added to the Environmental Protection Agency High Production Volume Challenge Program which identifies ingredients manufactured or imported into the United States in amounts equal to greater than one million pounds per year.

4 | UV ABSORPTION SPECTRUM, SUNSCREEN EFFICACY TESTING, AND SKIN CANCER RATES

With the recent attempts in Hawaii to ban the use of oxybenzone in sun protection factor (SPF) products, some have expressed concern over losing an effective UV absorber and possibly causing an increase in the number of skin cancers observed annually. The ability of a sunscreen product to protect against UV rays is not based on an individual ingredient contained in a formula, however, but rather how the formula performs, as a whole, when tested according to the Food & Drug Administration (FDA) guidelines for labeling and effectiveness testing; sunscreen drug products for over-the-counter human use.[2] For example, a product could contain the most effective UV-absorbing ingredients allowed (avobenzone, titanium dioxide, or zinc oxide), but if it is formulated in an inappropriate way that product would deliver little to no protection from the damaging effects of UV rays. This reality underlies why FDA has established these guidelines and requires as a matter of law that all formulas be tested for efficacy and stability prior to being sold in the marketplace. Therefore, any product sold in the United States that claims a SPF and, further, makes a broad spectrum claim—regardless of the ingredient(s) used in the product—can be trusted to perform according to the package labeling and protect against the carcinogenic effects of the sun.

It is important to note that SPF testing (UVB) is conducted in 10 human subjects, as outlined in the FDA testing guidelines. However, broad spectrum (UVA) testing is an analytical method (in vitro) that measures if a product has a critical wavelength of at least 370 nm, which represents 90 percent of the total area under the curve in the UV region. Based on the FDA definition, only zinc oxide, titanium dioxide, avobenzone, menthyl anthranilate, oxybenzone, and octocrylene would qualify out of all the approved actives noted in the sunscreen monograph (Table 1). It is important to note that based on these classification criteria, oxybenzone just makes the critical wavelength cutoff of at least 370 nm for UVA claims and would be tied for last place with octocrylene in terms of broad spectrum performance.

The average annual number of adults treated for skin cancer in the United States increased from 3.4 million in the 2002-2006 time period to 4.9 million annually between 2007 and 2011.[4] Correspondingly, the average annual total cost for managing skin cancer increased 126.2% from $3.6 billion to $8.1 billion. Importantly, the National Cancer Institute [5] consumer use data for adults aged 18 years or older between the years 2005 and 2015 report that 70.8% of all adults practice one of the three sun protective behaviors identified: (i) seeking shade and avoiding sun during peak hours (ii) wearing protective clothing; and (iii) using sunscreen. Of the three methods, only 33.7% reported applying sunscreens, while 38.4% relied on clothing and 39.1% usually sought shade.

Taking into account how products are tested for UV efficacy, the absorption spectrum of the currently approved FDA actives, and how

TABLE 1 Critical wavelength for commonly used UV filters with an attenuation of 370 nm and above[3]

FDA monograph sunscreen ingredients drug label name (INCI/Common Name)	Attenuation in NM	Peak absorption
Octocrylene	290-370	305-325
Oxybenzone (Benzophenone-3)	290-370	290-300 & 325-340
Menthyl anthranilate	290-380	340-350
Avobenzone (Butyl Methoxydibenzoylmethane)	290-390	355-370
Titanium dioxide	290-400	290-320
Zinc oxide	290-400	290-385

consumers actually deal with protecting against sun exposure, it is unclear that products containing oxybenzone offer any distinct benefits over other available options when it comes to reducing elevated epidemiological trends in skin cancer. Indeed, for those sunscreen users who have concerns and want the strongest UV protective against the sun, zinc oxide has the best UV attenuation (290-400 nm) and peak absorption (290-385 nm) of all actives, covering 100% of the UVB and 95% of the UVA spectrum. Zinc oxide can be used individually or with other actives, if UV protection above an SPF 30 is required for very sun sensitive individuals, and with the advent of micronized particles (100 nm or larger) product, esthetics are excellent and promote patience compliance. Lastly, it is more likely that patients would receive better protection from frequent application of sunscreens rather than solely relying upon higher SPF factors. For example, a product with a SPF 30 protects against 97% of UVB whereas a product with a SPF 50 protects against 98%; however, to gain that additional 1%, a SPF 50 product may contain almost twice the concentration of sunscreen actives, potentially increasing the chance of adverse reactions, particularly in patients with sensitive skin.

5 | SKIN REACTIVITY

In a study designed to describe allergens associated with a sunscreen source, the North American Contact Dermatitis Group evaluated both active and inactive ingredients in sunscreen products that may cause contact dermatitis. Standard patch testing in 23 908 patients was conducted between 2001 and 2010 and identified 219 (0.9%) positive reactions. The top three most frequent allergens in sunscreens were as follows: oxybenzone (70.2% for 10% concentration, 64.4% for 3% concentration), DL-alpha-tocopherol (4.8%), and fragrance mix I (4.0%).[6]

Similarly, the European Scientific Committee on Consumer Safety (SCCP) published an opinion paper[7] based on a review of 20 publications involving 6378 patients that were photo-patch tested for oxybenzone and other sunscreen actives between the 1981 and 2003. A total of 159 positive reactions were noted, leading to the conclusion that oxybenzone is a photoallergen. By way of comparison, only 19 photoallergic reactions were noted in these studies to p-aminobenzoic acid (PABA) and 34 photoallergic reactions to the various PABA esters.

Verhulst and Goossens[8] recently published a review and update of cosmetic products that have been reported to produce contact urticaria. Causative agents cited included phenoxyethanol, polyaminopropyl biguanide, oxybenzone, menthol, and a number of plant-derived ingredients including wheat and wheat protein hydrolyzates. Evidence of contact urticaria and, to a lesser degree, contact-mediated anaphylaxis was reported to be caused by oxybenzone.

The American Contact Dermatitis Society listed benzophenones as the 2014 Allergen of the Year, covering both allergy and photoallergy reactivity based on research reported by Heurung et al[9] They sighted oxybenzone (benzophenone-3) as the most frequent reactor in the class as well as the most prominent agent found in 68% of the 201 sunscreen products assessed. The authors also noted that oxybenzone showed high rates of cross-reactivity with the sunscreen active octocrylene, as well as ketoprofen, a topical nonsteroidal anti-inflammatory.

Additionally, oxybenzone has a molecular weight (MW) of 228.26 daltons, which raises concerns historically as a MW below 500 daltons has been associated with most of the common contact allergens.[10]

Cumulatively, there appears to be sufficient research demonstrating that oxybenzone possesses the potential to induce/elicit contact allergy, photocontact allergy, and contact urticaria reactions in humans. To put this into perspective, the sunscreen active p-aminobenzoic acid (PABA) and its esters have also been reported to produce allergic contact and photocontact dermatitis reactions[7,11] at somewhat lower reactivity rates than oxybenzone; however, PABA was forced into obscurity in the United States as a result of concerns from the medical community about sensitivity and subsequent competitive pressures on industry.

6 | ENVIRONMENTAL CONCERNS

In order to be effective, SPF products must be formulated to stay on the surface of the skin where they can reduce the penetration of UV energy to the underlying tissue. Therefore, when formulated in an effective SPF vehicle, oxybenzone demonstrates little absorption through the skin despite having a low MW. Gonzalez et al[12] observed an average excretion rate of 3.7% of the dose of a commercial sunscreen containing 4% oxybenzone when applied morning and night for 5 days. Accordingly, it is possible to estimate that if approximately 4% of oxybenzone in a sunscreen formulation is absorbed into the skin, 96% of the remaining dose is available to be washed off and enter various waterways. Corroborating this point, a 2008 study estimated that 4000 to 6000 tons of sunscreens were washed off in tourist reef areas annually[13]; as of 2017, scientists are currently estimating that 8000 to 16000 tons of sunscreen enter coral reefs each year.[14] The increase is the result of the continued growth of the global sunscreen market, which is projecting to reach sales of $11 billion by the year 2020. To better understand the implications of these figures, Tsui et al[15] sampled the waters of eight cities across four countries (China, United States, Japan, and Thailand) and the North American Arctic identifying twelve widely used aromatic hydrocarbon UV chemical filters. In general, concentrations of the chemicals increased with population density. Oxybenzone concentrations ranging from as high as 33 parts per trillion (ppt) in the Arctic to 5 parts per billion (ppb) in Hong Kong were identified. It should be noted that the surface waters sampled came largely from metropolitan areas featuring both commercial and industrial development, as opposed to beach or resort communities that see high levels of recreational water use by humans. Moreover, the concentrations in the Arctic waters suggest significant migration of toxic chemicals is occurring as current and tidal forces lead to water migration. The authors concluded that the findings represent various ecological risks to marine ecosystems, including promoting coral bleaching and adversely affecting reproduction in fish.

In 2008, Danovaro et al[13] were one of the first to report that oxybenzone had a negative impact on coral causing bleaching and death at concentrations of 33 and 50 parts per million (ppm). Additional research published in 2015 by Downs et al[16] identified oxybenzone as a phototoxicant, genotoxicant, and a skeletal endocrine disruptor in coral. They determined a lethal concentration 50 (LC50) for coral larvae that ranged from 139 to 3100 ppb depending on the specific test conditions. Coral cell LC50s for seven different coral species ranged from 8 to 340 ppb. The authors went on to measure the amount of oxybenzone at various locations (bays and open waters) at two different locations: Concentrations in the sampled waters from the U.S. Virgin Islands ranged from 75 to 1400 ppb and the Hawaiian Islands 0.8 to 19.2 ppb. Based on these findings, the water concentration of oxybenzone currently in the Virgin Islands overlaps the LC50 calculated for coral larvae and coral cells, while the waters in Hawaii are starting to reach levels that are within the range of the LC50 for coral.

The identification and accumulation of oxybenzone in waters cause concerns not just to coral, but to many other aquatic species as well. Braush and Rand [17] reviewed oxybenzone toxicity in Daphnia magna (invertebrate) and Oncorhynchus mykiss and Oryzias latipes (fish) and found LC50s of 1.9 ppm, 749 ppb, and 620 ppb, respectively. The authors further identified that UV filters have been shown to have bioaccumulation factors greater in fish than in water. For example, Gago-Ferrero et al[18] evaluated the accumulation of UV absorbers in a variety of fish in Spain and were able to extract oxybenzone from the tissue of white fish, rainbow trout, barb, chub, perch, and mussels. Taken together, these studies suggest the potential for increasing concentrations in species higher up in the trophic level, with humans poised to ingest the highest concentrations from the larger species that are regularly fished for human consumption.

UV filters enter the environment in two primary ways, directly from sloughing off while swimming around reefs or other waterways and indirectly via wastewater treatment plant (WWTP) effluent. In fact, even swimming in chlorinated pools and people washing sunscreens off their bodies while bathing raises several concerns. Researchers have observed that chlorine can react with oxybenzone producing chlorinated oxybenzone, which results in significantly more cell death than unchlorinated controls.[19] Another study evaluated oxybenzone transformation and kinetics after chlorination.[20] These results indicated that more genotoxic transformation products were produced in spite of the elimination of oxybenzone, posing potential threats to drinking water safety. Similarly, six water treatment plants in southeast Brazil evaluated WWTP levels of oxybenzone and observed (0.18 to 1.15 ppb) in both raw treated and chlorinated water, indicating that the compound was not removed by the water treatment process.[21] Additionally, Braush and Rand[17] reported that Switzerland estimated the input of 69 g of oxybenzone per 10,000 people per day into their WWTP.

The Centers for Disease Control and Prevention evaluated urinary samples obtained from 2517 participants aged 6 years and older between 2003 and 2010 and identified oxybenzone levels ranging from 15 ppb up to 3 ppm.[22] Meeker et al[23] recruited 105 pregnant women in northern Puerto Rico to provide urine samples and complete questionnaire data at three times during gestation. Urinary concentrations of oxybenzone ranged from 41.0 to 66.4 ppb and a positive association between biomarker concentrations, and self-reported use of personal care products was reported. An intraclass correlation coefficient (ICC) of 0.62 was determined for oxybenzone which was the highest among all the chemicals identified in the study. In contrast, urine samples were collected from 33 young Danish men over a 3-month period [24] with ICCs ranging from 0.69 to 0.80 and with more than 70% of the urine samples having detectable levels of oxybenzone. These data suggest that while most oxybenzone in personal care products is not significantly absorbed, sufficient quantities do enter the body such that meaningful levels can be measured in urine that finds its way to WWTP. Oxybenzone and/or its metabolite 4-methylbenzophenone may be more ubiquitous than generally thought (e.g., not just in sunscreens, cosmetics, and fragrances). The International Agency for Research on Cancer[27] has identified several sources of dietary exposure to these molecules in food or addition to food as a flavoring agent, its presence in drinking water as a contaminant, and through its migration from food packaging, printing inks, or recycled paperboard.

Kim and Choi[25] observed that oxybenzone has been detected in water, soil, sediments, sludge, and biota. Based on their review, the maximum detected level in ambient freshwater and seawater was 0.13 ppb and 0.58 ppb, respectively, and in wastewater, influent was 10.4 ppb. They also noted that in humans, oxybenzone has been detected in urine, serum, and breast milk samples worldwide with receptor binding assays showing strong adverse endocrine effects, including anti-androgenic and anti-estrogenic activity. Predicted no-effect concentration (PNEC) for oxybenzone was derived at 1.32 ppb; the levels observed in ambient water are generally an order of magnitude lower than the PNEC, but in wastewater influents, hazard quotients greater than 1 were noted. Lastly, Huo et al[26] looked at the relationship between maternal oxybenzone exposure and Hirschsprung's disease (HSCR) as well as its potential mechanism. HSCR is a neonatal intestinal abnormality that is derived from the failure of enteric neural crest cells migration to hindgut during embryogenesis from 5 to 12 weeks. The results showed that maternal oxybenzone exposure was associated with offspring developing HSCR, likely due to the chemical's inhibiting migration of highly specific cells.

7 | CONCLUSION

Based on the data reviewed, oxybenzone can be found globally in water, soil, sediments, sludge, and biota as well as in human urine, serum, and breast milk. As a sunscreen active, it is not as effective at protecting against UVA exposure as avobenzone, titanium dioxide, and/or zinc oxide. In humans, the chemical has been linked to Hirschsprung's disease is a confirmed contact allergen and photocontact allergen with some potential to induce contact urticaria and, to

a lesser degree, contact-mediated anaphylaxis. Environmentally, oxybenzone inhibits reproduction of coral and fish via embryo toxicity and/or causing male fish to be feminized, coral bleaching, and/or death. In summary, the potential negative health and environmental effects caused by the accumulation of this and other chemicals in the ecosystem needs to be taken into consideration by industry and regulatory agencies prior to the development and release of new and effective personal care products.

ORCID

Joseph C DiNardo http://orcid.org/0000-0001-8823-9814

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