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

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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 LC50 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 LC50s for oxybenzone in darkness for the same time points were 16.8 mg/L and 779 μg/L. Deformity EC20 levels (24 h) of planulae exposed to oxybenzone were 6.5 μg/L in the light and 10 μg/L in darkness. Coral cell LC50s (4 h, in the light) for the same species ranges from 0.062 to 8 μg/L. Coral reef
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-methoxyphenyl phenylmethanone; CAS No. 131-57-7) is often 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 ppttrillion (Tashiro and Kameda 2013); in South America, sediments near coral communities/reefs contained BP-3 concentrations between 54 and 578 ppttrillion (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 asp 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 (LC50), effect concentration (EC20), 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 LC50 results of BP-3-exposed planulae to the BP-3 LC50 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. Benzenophene-3 (BP-3; 2-Hydroxy-4-methoxyphenyl-phenylmethane; 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 planulae 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 irradiance of 288 photosynthetic photon flux density μmol/m²/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-Teflon 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/m²/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-
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 pptillion, 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 (EC20 and EC50) and median Lethal Concentration response (LC50) 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 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 pptillion, and Quantitative Limit of Measurement was 5 ppbillion (μg/L).

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Data were tested for normality (Shapiro–Wilks 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
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
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 3f 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 chloroplast.
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; Eskelinnin 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. 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.
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.

Transmission electron microscopy of planulae exposed to 228 parts per billion (μg/L) benzophenone-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. 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.

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 autophagosomes, 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
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 CaCO₃ 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 CaCO₃ 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 \( \mu \)g/L (H Statistic = 21.903; \( P < 0.001 \); Dunnett’s Procedure). The NOEC for planulae

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**Fig. 5** Transmission electron microscopy of *Stylophora pistillata* planula exposed to 228 parts per billion (\( \mu \)g/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.
in the dark determined by a Kruskal–Wallis One-Way Analysis of Variance on Ranks was 228 µg/L (H Statistic = 22.402; P < 0.001; Dunnett’s Procedure). 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 < 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 < 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$_{50}$ 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 (mg/L), whereas LC$_{50}$ in the dark was 12.811 mg/L (mg/L) for cells exposed to the light for 4 h (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 < $R^2$ < 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).
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
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
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 Mau-nalua 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 ppbillion; 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 Kapalua 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).

### 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 \text{mol/m}^2/\text{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 1

Regression and PROBIT determination of LC50 for planulae mortality when exposed to BP-3 in the light and dark, and the EC50 for planulae deformity when exposed to BP-3 in the light and the dark

<table>
<thead>
<tr>
<th>Planulae mortality</th>
<th>LC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression to estimate LC50 8-h light</td>
<td>3.1 mg/L</td>
</tr>
<tr>
<td>PROBIT to estimate LC50 8-h light</td>
<td>2.9 mg/L</td>
</tr>
<tr>
<td>Regression to estimate LC50 8-h dark</td>
<td>16.8 mg/L</td>
</tr>
<tr>
<td>PROBIT to estimate LC50 8-h dark</td>
<td>12.8 mg/L</td>
</tr>
<tr>
<td>Regression to estimate LC50 24-h light</td>
<td>103.8 µg/L</td>
</tr>
<tr>
<td>PROBIT to estimate LC50 24-h light</td>
<td>1.39 µg/L</td>
</tr>
<tr>
<td>Regression to estimate LC50 24-h dark</td>
<td>873.4 µg/L</td>
</tr>
<tr>
<td>PROBIT to estimate LC50 24-h dark</td>
<td>799 µg/L</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Planulae deformation</th>
<th>EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression to estimate EC50 8-h light</td>
<td>107 mg/L</td>
</tr>
<tr>
<td>PROBIT to estimate EC50 8-h light</td>
<td>133 mg/L</td>
</tr>
<tr>
<td>Regression to estimate EC50 8-h dark</td>
<td>436 mg/L</td>
</tr>
<tr>
<td>PROBIT to estimate EC50 8-h dark</td>
<td>737 mg/L</td>
</tr>
<tr>
<td>Regression to estimate EC50 24-h light</td>
<td>17 µg/L</td>
</tr>
<tr>
<td>PROBIT to estimate EC50 24-h light</td>
<td>49 µg/L</td>
</tr>
<tr>
<td>Regression to estimate EC50 24-h dark</td>
<td>105 µg/L</td>
</tr>
<tr>
<td>PROBIT to estimate EC50 24-h dark</td>
<td>137 µg/L</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Planulae deformation</th>
<th>EC20 (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROBIT to estimate EC20 8-h light</td>
<td>6.3</td>
</tr>
<tr>
<td>PROBIT to estimate EC20 8-h dark</td>
<td>15.5</td>
</tr>
<tr>
<td>PROBIT to estimate EC20 24-h light</td>
<td>6.5</td>
</tr>
<tr>
<td>PROBIT to estimate EC20 24-h dark</td>
<td>10.4</td>
</tr>
</tbody>
</table>

### Table 2

Differences in sensitivities of various species of Indo-Pacific and Caribbean coral reefs, the LC50s and LC20s 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

<table>
<thead>
<tr>
<th>Coral species</th>
<th>LC50 (µg/L)</th>
<th>95 % CI</th>
<th>LC20</th>
<th>95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Indo-Pacific species</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stylophora pistillata (light)</td>
<td>42</td>
<td>28; 60</td>
<td>2 µg/L</td>
<td>1.14; 3.61</td>
</tr>
<tr>
<td>Stylophora pistillata (dark)</td>
<td>671</td>
<td>447; 984</td>
<td>14 µg/L</td>
<td>7; 26</td>
</tr>
<tr>
<td>Pocillopora damicornis</td>
<td>8</td>
<td>4.96; 12.15</td>
<td>62 ng/L</td>
<td>24; 136</td>
</tr>
<tr>
<td><strong>Caribbean-Atlantic species</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acropora cervicornis</td>
<td>9</td>
<td>5.4; 14.5</td>
<td>63 ng/L</td>
<td>22; 150</td>
</tr>
<tr>
<td>Montastrea annularis</td>
<td>74</td>
<td>40; 126</td>
<td>562 ng/L</td>
<td>166; 1459</td>
</tr>
<tr>
<td>Montastrea cavernosa</td>
<td>52</td>
<td>36; 72</td>
<td>502 ng/L</td>
<td>247; 921</td>
</tr>
<tr>
<td>Porites astreoides</td>
<td>340</td>
<td>208; 534</td>
<td>8 µg/L</td>
<td>3; 16</td>
</tr>
<tr>
<td>Porites divaricata</td>
<td>36</td>
<td>21; 57</td>
<td>175 ng/L</td>
<td>60; 420</td>
</tr>
</tbody>
</table>
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 “sun-screens 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. 2013).
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 Billinghamurst 1999). Surviving planulae exposed to BP-3 may settle, metamorphose, and

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.
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 Billinghamurst 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; Ziółkowski 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-mono- tonic.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 ppbillion 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 LC50s of coral cells in the light (42 ppbillion; µg/L) and coral planula in the light for 8 and 24 h [2.876 ppmillon (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 LC50 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 (synonymy 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 recruitment). These demographic patterns in the reproductive biology of scleractinian corals. 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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