Ecotoxicology (2014) 23:175–191
DOI 10.1007/s10646-013-1161-y

Toxicological effects of the sunscreen UV filter, benzophenone-2, on planulae and in vitro cells of the coral, *Stylophora pistillata*

C. A. Downs · Esti Kramarsky-Winter · John E. Fauth · Roee Segal · Omri Bronstein · Rina Jeger · Yona Lichtenfeld · Cheryl M. Woodley · Paul Pennington · Ariel Kushmaro · Yossi Loya

Accepted: 7 December 2013 / Published online: 19 December 2013

Abstract Benzophenone-2 (BP-2) is an additive to personal-care products and commercial solutions that protects against the damaging effects of ultraviolet light. BP-2 is an "emerging contaminant of concern" that is often released as a pollutant through municipal and boat/ship wastewater discharges and landfill leachates, as well as through residential septic fields and unmanaged cesspits. Although BP-2 may be a contaminant on coral reefs, its environmental toxicity to reefs is unknown. This poses a potential management issue, since BP-2 is a known endocrine disruptor as well as a weak genotoxicant. We examined the effects of BP-2 on the larval form (planula) of the coral, *Stylophora pistillata,* as well as its toxicity to in vitro coral cells. BP-2 is a photo-toxicant; adverse effects are exacerbated in the light versus in darkness. Whether in darkness or light, BP-2 induced coral planulae to transform from a motile planktonic state to a deformed, sessile condition. Planulae exhibited an increasing rate of coral bleaching in response to increasing concentrations of BP-2. BP-2 is a genotoxicant to corals, exhibiting a strong positive relationship between DNA-AP lesions and increasing BP-2 concentrations. BP-2 exposure in the light induced extensive necrosis in both the epidermis and gastrodermis. In contrast, BP-2 exposure in darkness induced autophagy and autophagic cell death. The LC_{50} of BP-2 in the light for an 8 and 24 h exposure was 120 and 165 parts per billion (ppb), respectively. The LC_{50}s for BP-2 in darkness for the same time points were 144 and 548 ppb. Deformity EC20 levels (24 h) were 246 parts per trillion in the light and 9.6 ppb in darkness.

Electronic supplementary material The online version of this article (doi:10.1007/s10646-013-1161-y) contains supplementary material, which is available to authorized users.

C. A. Downs (✉)
Haereticus Environmental Laboratory, P.O. Box 92, Clifford, VA 24533, USA
e-mail: cadowns@haereticus-lab.org

E. Kramarsky-Winter · R. Segal · O. Bronstein · Y. Loya
Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, 69978 Tel Aviv, Israel

E. Kramarsky-Winter · Y. Lichtenfeld · A. Kushmaro
Avram and Stella Goldstein-Goren Department of Biotechnology Engineering, Faculty of Engineering Sciences and National Institute For Biotechnology in the Negev, Ben-Gurion University of the Negev, 84105 Beer Sheva, Israel

J. E. Fauth
Department of Biology, University of Central Florida, 4000 Central Florida Boulevard, Orlando, FL 32816-2368, USA

R. Jeger
Department of Life Sciences, Ben-Gurion University of the Negev, 84105 Beer Sheva, Israel

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

C. M. Woodley · P. Pennington
Center for Coastal Environmental Health and Biomolecular Research, U.S. National Oceanic & Atmospheric Administration, 219 Ft. Johnson Rd., Charleston, SC 29412, USA

A. Kushmaro
School of Materials Science & Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798, Singapore

Keywords Coral · Benzophenone-2 · Cell toxicity · Coral planula · Sunscreen UV filters

Introduction

Benzophenone-2 (BP-2; 2,2′,4,4′-tetrahydroxybenzophenone, CAS No. 131-55-5) is a chemical additive in product coatings (oil-based paints, polyurethanes), acrylic adhesives, plastics and personal-care products that is used to mitigate the adverse effects of ultraviolet light exposure [Harper and Petrie 2003; CIR (Cosmetic Ingredient Review) 2005; Shaath and Shaath 2005]. A number of personal-care products may contain BP-2, such as bath salts, body fragrances and lotions, shampoos, as well as soaps and some laundry detergents [e.g., CIR (Cosmetic Ingredient Review) 2005]. BP-2 and other benzophenone derivatives are often found as contaminants in residential and municipal wastewater effluents, and are considered "emerging environmental contaminants of concern" by the U.S. Environmental Protection Agency (Daughton 2002; Eichenseher 2006; Richardson 2006, 2007; Gago-Ferrero et al. 2011; Rodil et al. 2012; Aquero et al. 2013). In the last 40 years, coastal development near coral reefs has increased exponentially, while modernization of waste-water management and scientific research of watersheds that directly impact coral reefs is alarmingly absent. This poses serious issues regarding BP-2-laden waste-water impacts on coral reefs, since both the threat of this chemical to coral reef ecological integrity and the extent of its pollution on reefs are unknown (Daughton 2002; Blitz and Norton 2008; http://www.epa.gov/ged/coralreef/models/SunscreenUse.html).

BP-2 exhibits a number of toxicological behaviors that can be observed from the molecular level to multi-organ system pathologies. Benzophenones, and BP-2 especially, are documented mutagens which increase the rate of damage to DNA under alkaline conditions (e.g., pH 8.2—pH of seawater) or when exposed to sunlight [Popkin and Prival 1985; Zeiger et al. 1987; Knowland et al. 1993; NTP (National Toxicology Program) 2006]. 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, such as inducing estrogen-associated cell proliferation of breast cancer cells (Kerdivel et al. 2013).

In addition to its genotoxicity, BP-2 is an endocrine disruptor, affecting a variety of tissue types and organ systems [Jarry et al. 2004; NTP (National Toxicology Program) 2006; Gilbert et al. 2013]. In mammals, BP-2 causes birth defects via an estrogen dependent mechanism (Schlumpf et al. 2004, 2008; Hsieh et al. 2007). In fish and mammals, BP-2 induces a variety of reproductive disorders, including feminization of male fish, inhibition of gamete development in fish, reduction of testosterone secretions from testicular tissue, induction of uterotrophic effects in rats, changes in bone density and osteo-regulation, changes in luteinizing hormone, cholesterol levels, fat deposition, and an increased risk of endometriosis (Seidlova-Wuttke et al. 2004; Koda et al. 2005; Kunz et al. 2006; Weisbrod et al. 2007; Kunz and Fent 2009; Kim et al. 2011; Kunisue et al. 2012). These pathologies are thought to stem from at least three distinct mechanisms: (1) BP-2 mimics the action of estrogen and activates estrogen receptor signal pathways, (2) BP-2 suppresses expression of enzymes in the production of testosterone, and (3) BP-2 reduces thyroid hormone levels (Schlumpf et al. 2001; Yamasaki et al. 2003; Seidlova-Wuttke et al. 2004, 2005; Jarry et al. 2004; Suzuki et al. 2005; Morohoshi et al. 2005; Schlecht et al. 2006; Molina-Molina et al. 2008; Nashez et al. 2010; Ye et al. 2011; Kim et al. 2011; Cosnefroy et al. 2012).

Jarry and co-workers (2004) first demonstrated that exposure of BP-2 in rats reduced triiodothyronine (T3) and thyroxine (T4) circulation. Thyroid hormones (THs) play essential roles in early stages of development, including influences of cellular proliferation, cell differentiation, cell migration, and neural development. In cnidarians (i.e., jellyfish, coral) and other invertebrates, thyroxines are required for planula metamorphosis and settlement (e.g., Spangenberg 1971; Burke 1983; Bishop et al. 2006). In rats, BP-2 interfered with the thyroid hormone axis and inhibited thyroid peroxidase activity, a heme-containing glycoprotein that transfers iodine to thyroglobulin during thyroid hormone synthesis (Schmutzler et al. 2007; Thienport et al. 2011; Song et al. 2012). Concentrations at parts per trillion (ppt) of BP-2 were able to reduce thyroid peroxidase in an in vitro assay by more than 80 % (Song et al. 2012).

Coral reefs are being degraded worldwide, and though regional weather and climate events can result in the mass-mortality of coral reef organisms, the long-term causative processes of sustained demise are often locality specific (e.g., Edinger et al. 1998; Rees et al. 1999; Golbuu et al. 2008; Smith et al. 2008; Downs et al. 2011, 2012; Omori 2011). This is most apparent in the severe decline of juvenile coral recruitment and survival rates along coastal areas that are densely populated by humans (e.g., Dustan 1977; Miller et al. 2000; Hughes and Tanner 2000; Abelson et al. 2005; Williams et al. 2008). As with other invertebrate species, coral larval development is more sensitive to the toxicologic effects of pollution as compared to adults. Hence, even small impacts to larval development and

survival can have significant effects on coral demographics and community structure (Richmond 1993, 1997). In order to better manage BP-2 pollution and its effect on the ecological resilience of coral reefs, the toxicological effects of BP-2 on larval survival and development need to be characterized.

In this study, we examined the toxicologic effects of BP-2 exposures of varying concentrations on the larval form (planula) of the scleractinian coral *Stylophora pistillata*. Many chemical pollutants affect organisms differently when exposed to light, a process known as chemical-associated phototoxicity (Yu 2002; Platt et al. 2008). Since 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-2 exposures in planulae subject to either darkness or to environmentally-relevant light conditions. Histopathology and cellular pathology, planula morphology, coral bleaching, DNA damage as formation of DNA abasic lesions, and planula mortality were measured in response to BP-2 exposure. Median lethal concentration (LC_{50}), effect concentration (EC_{20}) and no observable effect concentrations (NOEC) were determined for coral planulae exposed to BP-2 in both darkness and in the light. Coral planulae are an extremely difficult resource to procure for toxicological studies. Therefore, primary coral cell cultures were used in in vitro toxicological tests of BP-2 to examine their validity as a surrogate model for coral planula 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-2-exposed planulae to the BP-2 LC_{50} of coral cells (calicoblasts) from adult *S. pistillata* colonies.

Materials and methods

Planula collection and toxicity exposures

Planulae 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. Traps were set between 17:00 and 18:00 h, then retrieved at 06:00 h the next day. Planulae were inspected and sorted by 07:15 h, and toxicity exposure experiments began at 08:00 h.

Exposures were conducted in virgin-PTFE-Teflon 24-well microplates. Ten planulae were placed in each well, while four wells (i.e., four replicates) in each 24-well microplate were used for each treatment concentration. Microplates were placed in a natural seawater flow-through tank system to regulate a constant temperature (22 °C). Lighting was natural sunlight filtered with neutral density filters for a maximum irradiance at 13:08 h of 406 μmol m^{-2} s^{-1} of photosynthetic active radiation.

All seawater (ASW) was made artificially using Fisher Scientific Environmental-Grade water (catalog #W11-4) and Sigma-Aldrich sea salts (catalog #S9883) to a salinity of 38 parts per thousand at 22 °C. Benzophenone-2 (BP-2; 2,2′4,4′-tetrahydroxy benzophenone; Aldrich catalog #T16403) was solubilized in dimethyl sulfoxide (DMSO), then diluted with ASW to generate stock solutions and exposure solutions. Solutions of BP-2 for toxicity exposures each contained 5 μL of DMSO per one liter and were of the following concentrations: 1 mM BP-2 [246 parts per million (ppm)], 0.1 mM BP-2 (24.6 ppm), 0.01 mM BP-2 (2.46 ppm), 0.001 mM BP-2 (246 ppm), 0.0001 mM (24.6 ppm), and 0.00001 mM (2.46 ppm). For every exposure time-period, there were two controls with four replicates each: (a) planulae in ASW, and (b) planulae in ASW with 5 μL of DMSO per one liter.

Planulae were exposed to the different BP-2 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. Each well contained 2.5 mL of ASW/BP-2 solution. In response to well-evaporation during daylight hours, salinity was maintained at 38 parts per thousand using Fisherbrand pure water. For the 24-h exposure, planulae were transferred to new 24-well microplates with fresh ASW/BP-2 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, planular 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 apurinic/apyrimidinic (AP) site assay.

Chlorophyll fluorescence as an estimate for coral bleaching

In the literature, bleaching is often described anecdotally and rarely using repeatable quantification methods. The best quantifiable methods for bleaching are “destructive” methods that require an abundance of tissue material, measuring either total chlorophyll per area or total number of zooxanthellae per area (zooxanthella = commensal symbiotic dinoflagellate of coral). Neither of these methods were feasible for these experiments with coral planulae.

There was insufficient chlorophyll per planula (in controls) to validly use the chlorophyll acetone/spectrophotometric method (Lichtenthaler 1987). Zooxanthella cell counts, using a microscopic cell-counting chamber, resulted in high variance because of low numbers per planula, and the inability for accurate discrimination. Zooxanthellae in BP-2-treated planulae were also very susceptible to rupturing during the isolation method. As an alternative to these two methods, chlorophyll content can be measured via a non-destructive chlorophyll fluorescence method (Agati et al. 1995; Gitelson et al. 1999). However, because planulae are three dimensional, and have a rod-like or spheroid-like shape, planar incidence of fluorescence emission is an artifactual measurement; it does not reflect the total surface area of the planula. Hence, measurement of chlorophyll fluorescence in planulae as a representation of coral bleaching is a gross estimation. Taking into consideration this caveat, chlorophyll fluorescence was measured using a Molecular Dynamics microplate fluorimeter with an excitation wavelength of 445 nm and an emission wavelength of 685 nm. Fluorescence measurements were taken at the end of the 8-hour light and dark periods of BP-2 exposure.

DNA abasic lesions

DNA abasic or apurinic/apyrimidinic lesions (DNA AP sites) were used as an indicator of genetic damage. DNA was isolated from frozen planulae according to the manufacturer's instructions using the Dojindo Get pureDNA Kit-Cell, Tissue (catalog #GK03-20; Dojindo Molecular Technologies, Inc., Rockville, MA, USA) with one slight modification to address Maillard chemistry artifact. The frozen planulae were added to the kit's lysis buffer containing 10 mg of polyvinylpolypyrrolidone (PVPP, Sigma-Aldrich Corporation, St. Louis, MI, USA). DNA purity was determined spectrophotometrically using the 260/280 nm method (Sambrook and Russell 2001). The DNA concentration was measured using an Invitrogen/Molecular Probes Quant-iT^{TM} DNA Assay Kit, Broad Range (catalog # Q33130; Life Technologies Corporation, Grand Island, NY, USA) using a Bio-Tek FL800 fluorescent microplate reader (BioTek Industries, Incorporated, Winooski, VT, USA). DNA AP sites were quantified using the Dojindo DNA Damage Quantification Kit-AP Site Counting (catalog # DK-02-10; Dojindo Molecular Technologies, Inc.) and conducted according to the manufacturer's instructions.

Transmission electron microscopy

Transmission electron microscopy was used for tissue and cellular pathomorphology assessment. For primary fixation, the planula sample was submerged in modified Karnovsky's fixative (2.5 % gluteraldehyde, 2 % paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) for 30 min and then transferred to a solution of 2.5 % gluteraldehyde in filtered seawater. Samples were fixed overnight in this secondary fixative. Planula samples were washed twice in 0.1 M cacodylate buffer (pH 7.2), and post-fixed in 1 % osmium tetroxide 4 °C for 30 min to enhance membrane preservation. Samples were then decalcified by repeated washes in Na_2EDTA. Samples were dehydrated in a graded ethanol series, then in propylene oxide followed by gradual embedding in Araldite (Catalog #502; Electron Microscopy Sciences). Samples in the final, full-strength araldite were subjected to a mild vacuum (400 mbar) for 1 h at 25 °C followed by overnight polymerization at 60 °C. The resulting block was first trimmed and 1 µ sections were cut and stained with toluidine blue to inspect the quality and orientation of the tissue (Carson 1997). The block was then sectioned (60–90 nm) using an ultramicrotome and sections mounted on 300 mesh copper grids. Ultrathin sections were stained with lead citrate. Sections through approximately the same mid-polyp body area were examined using a JOEL JEM-1230 at 80 kV transmission electron microscope and images photodocumented with a TVIPS TemCam-F214 (Tietz Video and Image Processing System, Germany).

Coral cell toxicity assay

Stylophora pistillata colonies were obtained from Exotic Reef Imports (www.exoticreefimports.com). Corals were maintained in glass and Teflon-plumbed aquaria in 36 parts per thousand 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 was measured using a Licor 250A light meter and planar incidence sensor.

Using surgical bone cutters, the coral was cut into 1 cm long pieces. Using Breaker-Grozier pliers, coral tissue and its underlying skeleton were scraped from the surface of the fragment to a depth of about 3 mm, thereby avoiding inclusion of endolithic algae or sponge. The resulting bolus of coral tissue was placed in a glazed ceramic dish containing 30 mL of artificial seawater (same composition as used in coral planula experiment) containing 3 unit/mL of lysozyme, 2 unit/mL of α-amylase, 0.5 unit/mL of α-glucosidase and β-galactosidase, and 0.25 unit/mL of endoglycosidase H at 24 °C for 10 min. After this incubation at least 2 units/mL of dispase was added to the coral suspension and incubated for 10 min. The bolus was gently mixed into solution using a stainless steel whisk every 2 min, and then allowed to incubate on a rocking platform for an additional 10 min. The cell suspension was aspirated

from the bowl and placed into 15 mL borosilicate glass centrifuge tubes. The tubes with cell suspension were centrifuged for 3 min at 65×*g*, after which the supernatant was transferred to clean 15 mL glass centrifuge tubes, and recentrifuged for 5 min at 88×*g*. This differential centrifugation process was repeated once more, and the final pellet was resuspended in ASW containing 3 unit/mL of lysozyme, 2 unit/mL of α-amylase (Downs et al. 2010).

A Percoll stock base (100 % Percoll Solution; Sigma-Aldrich) was made at 25 °C, consisting of 50 mL of Percoll, 5 gm of Ficoll 400, 3 gm of PEG 8000, 3 unit/mL of lysozyme, and 2 unit/mL of α-amylase. Percoll solutions of 20, 40, and 80 %, were created by diluting the 100 % Percoll solution to the appropriate concentration using ASW. After each of the three Percoll solutions were made, solutions were refrigerated at 5 °C for at least 30 min before construction of the step gradients. A Percoll-step gradient consisting of 3 mL per Percoll concentration of 20, 40, and 80 % was formed in a 15 mL, 1-cm diameter nitrocellulose centrifuge tube. Two milliliters of cell suspension was layered on top of the gradient, and centrifuged at 150×*g* for 6 min. Cells were collected at the 20 % Percoll density zone, diluted in five volumes of ASW, centrifuged at 700×*g* for 5 min. The supernatant was aspirated, and the cells resuspended in a coral cell-culture media (pH 8.2) consisting of filter-sterilized ASW (Sigma-Aldrich catalog# S9883; 36 parts per thousand salinity), 1 mL RPMI-1640 100× vitamin solution to 99 mL of ASW, 50 mM Hepes/KOH (pH 8.2), 1 mM calcium chloride, 1 mM sodium pyruvate, 0.075 g/L D-glucose, 0.3 g/L galactose, 0.25 g/L DL Na lactic acid, 0.25 mM ascorbate, 0.05 g/L α-lipoic acid, 0.5 mM proline, 0.5 mM cysteine, 0.5 mM methionine, 0.01 mM hydroxycobalamin, 0.001 mM Na folate, 1 g/L bovine albumin (V), 0.05 g/L succinate, and 0.25 g/L L-glutamine. Cells were cultured for 48 h, with media changes every 8 h. For cell culturing, cells were counted using a a modified Neubauer Hemocytometer (Hausser-Levy Counting Chamber) and suspended to a density of 242,000 cells per 1 mL in coral cell-culture media.

Exposure experiments of cells were conducted in PTFE-Telfon microplates. Cells were exposed to BP-2 concentrations of 615 ppt to 246 ppm for four hours either in the light or in the dark. 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 photosynthetic photon flux density.

Viability was confirmed using the trypan blue exclusion assay. Duplicate cells in a replicated 24-well Teflon plate seeded with the same number of cells per well and exposed to the same treatments were aspirated from each well, centrifuged at 300×*g* for 5 min, and the supernatant aspirated. Cells were gently resuspended in culture media that contained 1.5 % (w/v) of filtered trypan blue (Sigma-Aldrich, catalog #T6146), and incubated for 5 min. Viable versus dead cells were counted using a modified Neubauer Hemocytometer (Hausser-Levy Counting Chamber).

Statistical methods

To address different philosophies and regulatory criteria, effect concentration response (EC_{20} & 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 (cf. Crawley 1993), as well as having good fit, statistically significant and biologically interpretable regressors (Agresti 2002; Newman 2013). In several analyses, BP-2 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 both the Steel Method and the 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 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 made results of this test less powerful. To facilitate comparisons among other treatment means, figure legends include results of Tukey's Honestly Significant Difference Test or Student–Newman–Keuls 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. Since coral planulae are available only immediately after planulation, 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, USA) and SigmaPlot 12.0 was used for all analyses.

Results

Pathology

Within the first two hours of exposure in both light and darkness, planulae at all concentrations of BP-2 began to manifest a change from a normal, elongated morphology (Fig. 1a) that constantly moved through the water column by ciliary action to a deformed "dewdrop" or spherical form with significantly reduced movement (Fig. 1b). By the fourth hour of exposure for all BP-2 concentrations in the light and dark, movement ceased for all deformed planulae and the oral pole began to increase in diameter at least four to fivefold compared to controls, while no change was seen in the aboral pole of the planulae (Fig. 1b). By the end of 8 h of exposure for all BP-2 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.

Anecdotally, planulae exhibited signs of increased bleaching (i.e., loss of zooxanthellae, photosynthetic pigments, or both) with increasing concentrations of BP-2, both for the 8-h exposures, as well as the 24-h exposures. Reduced chlorophyll fluorescence corroborated these observations (Fig. 2).

Planulae exposed to BP-2 in the light experienced catastrophic tissue lysis both within the epidermis and gastrodermis (Fig. 3 vs 4). Cellular necrosis was the dominant form of cell death in both tissues, though some instances of autophagy and autophagic cell death were apparent in non-necrotized cells (Fig. 4d, Tsujimoto and Shimizu, 2005; Samara et al. 2008). The classic signs of apoptosis were not observed in any cell or tissue type (Kerr et al. 1972; White and Cinti 2004; Taatjes et al. 2008). Cells within the gastrodermis predominantly exhibited necrosis, with no signs of symbiophagy (Fig. 4e; Downs et al. 2009). The zooxanthellae within the planulae exhibited signs of necrosis and severe suborganellar catastrophic structural failure (Fig. 4e, d). Chloroplasts disintegrated into cellular debris while the nucleolus material disappeared in the micrographs and darkly-stained inclusion bodies appeared. Zooxanthella cell death did not occur via a mediated cellular process, but through catastrophic collapse of intracellular morphology, often associated with massive photo-oxidative stress (Downs et al. 2013). Coral bleaching resulted from both chloroplast degradation *in hospite* zooxanthellae and from the complete loss of zooxanthellae.

Planulae exposed to BP-2 in darkness exhibited distinct pathologies in both the epidermis and gastrodermis in contrast to planulae exposed to BP-2 in the light. Ciliated cells in the epidermal layer disappeared; the mechanism of death for this cell type is not clear from the electron micrographs (Fig. 5a). The majority of other cell types in the epidermis exhibited classic signs of autophagic cell death (Fig. 5a–c; Samara et al. 2008; Tasdemir et al. 2008; Yla-Antilla et al. 2009; Eskelinin et al. 2011). All cells showed an abundance of vacuolated bodies (autophagosomes), while unique cell structures, such as nematocytes, exhibited extensive vacuolization (Fig. 5b). As with BP-2 light exposures, there were no indications of the classic signs of apoptosis, though there were clear signs of autophagic cell death, such as vacuolated nuclei (Fig. 5c; Klionsky et al. 2012). The extent of autophagy in the gastrodermis mirrored that of the epidermis (Fig. 5d). Zooxanthellae within gastrodermal cells were experiencing symbiophagy; extensive vacuolization around the zooxanthella was apparent in all cases (Fig. 5d; Downs et al. 2009). Within the zooxanthellae, signs were apparent for

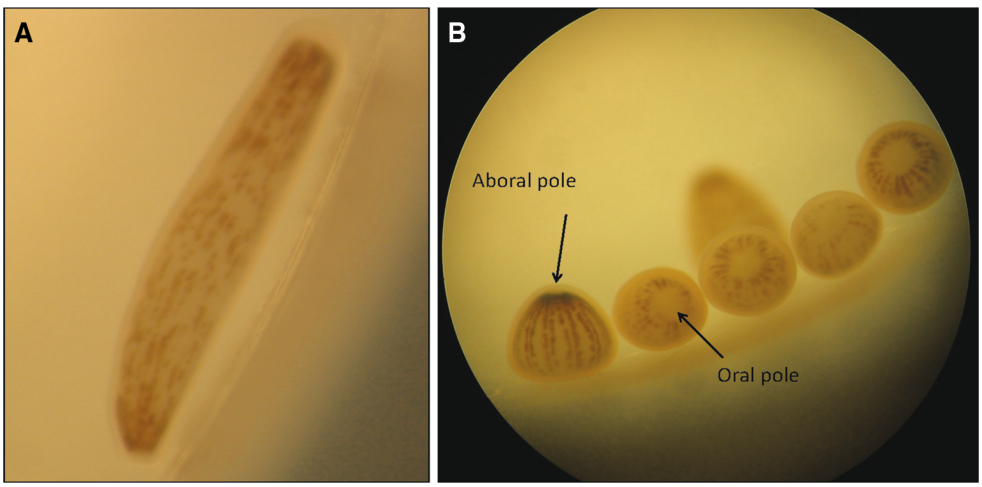

Fig. 1 Planula of *Stylophora pistillata* exposed to various treatments of benzophenone-2 (BP-2). **a** control planulae exposed for 8 h in light. **b** planulae exposed to 24.6 ppm BP-2 for 8 h in the light

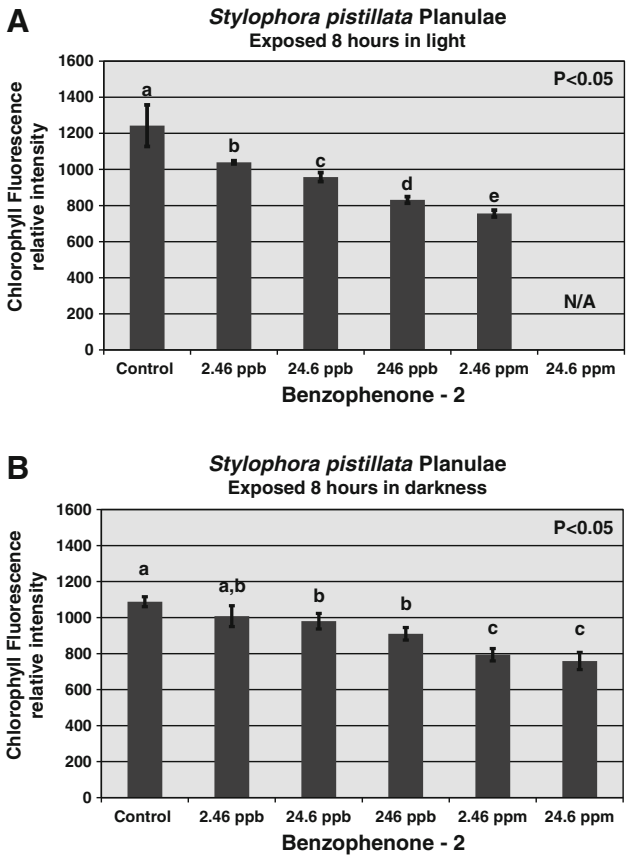

Fig. 2 Relative chlorophyll fluorescence emission at 685 nm with excitation at 445 nm of *Stylophora pistillata* planulae exposed to various treatments of benzophenone-2 (BP-2). *Bars* show treatment means with whiskers representing ±1 standard error of the mean. **a** planulae exposed to various BP-2 concentrations for 8 h in the light. Treatment means with *different superscript 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. **b** planulae exposed to various BP-2 concentrations for 8 h in the dark. Treatment means with *different superscript letters* differed significantly at $P < 0.05$, based on one-way analysis of variance followed by a Student–Newman–Keuls post hoc test

both organellar autophagy and unmediated cellular-degradation (Fig. 5e, f). Mitochondria within the zooxanthellae exhibited the classic hallmarks of autophagosome transformation (Fig. 5e). Chloroplasts exhibited a number of distinct pathologies, most likely the result of a process of chloroplast deterioration. A number of chloroplasts presented early stages of thylakoid lamellae fusion (Fig. 5f, labeled as "t"), while others showed inclusions resulting from biphasic separation of the trilayer thylakoid membrane (Fig. 5e, f, labeled as "i"; Downs et al. 2013).

In contrast to the findings of Danovaro et al. (2008), viral inclusion bodies were not observed in our electron microscopy examination.

Increasing concentrations of BP-2 induced significantly higher levels of DNA AP lesions in planulae exposed to the light compared to the controls (Fig. 6a). The V_{max} of the rate of lesion formation occurred at 24.6 ppm BP-2, most likely a result of the collapse of the DNA glycosylase/AP endonuclease pathway (Kuba et al. 1992; Wilson and Barsky 2001). DNA AP lesions were markedly higher in planulae exposed to BP-2 concentrations in the dark (Fig. 6c).

No-observed-effect levels

No-observed-effect levels (NOELs) for planulae exposed to BP-2 for 8 h were difficult to estimate because responses in the control were homogeneous; all planulae survived in some treatments and were not deformed (Fig. 7, 8). Analyses therefore required the less powerful nonparametric Steel Method. The NOEL for both the proportion of live coral planulae and non-deformed planulae exposed for 8 h in either the light or the dark was 246 ppm (1,000 μM) (all $Z > 2.37$ $P < 0.0716$). In contrast, the NOEL for DNA AP sites in planulae met ANOVA assumptions (Fig. 6), and was determined as 246 ppb (1,000 nM; one-way ANOVA $F_{5,18} = 147.2$, $P < 0.0001$, $R^2 = 0.98$; Dunnett's procedure for this comparison, $P < 0.0001$) when exposed in the dark, and 24.6 ppb (100 nM) when exposed in the light (one-way ANOVA $F_{5,18} = 69.2$, $P < 0.0001$, $R^2 = 0.95$; Dunnett's procedure for this comparison, $P < 0.0001$).

No-observed-effect levels (NOELs) for planulae exposed to BP-2 for 24 h were difficult to estimate because responses in the control and in all concentrations ≥ 10,000 nM were homogeneous; all planulae survived and were not deformed in the control, but died at the higher concentrations (Figs. 7, 8). Using the nonparametric Steel Method, we determined the NOEL as 246 ppb (1,000 nM) for all 24 h exposures to BP-2 (all $Z = 2.48$ $P = 0.0543$), regardless of whether exposures occurred in the light or dark.

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

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 exposure to BP-2 had coefficients of determination (R^2) between 0.81 and 0.98. Using a quadratic regression, the LC_{50} for the proportion of live coral planulae exposed in the light was 487,369 nM (120 ppm), while planulae exposed in the dark, the LC_{50} was slightly higher: 144 ppm (585,730 nM) (Supplemental Fig. 1a, c). PROBIT analysis for LC_{50} in the light was 28.315 ppm (114,998 nM), while LC_{50} in the dark was 155.9 ppm (633,174 nM) (Supplemental Fig. 5a, c). The EC_{50} for non-

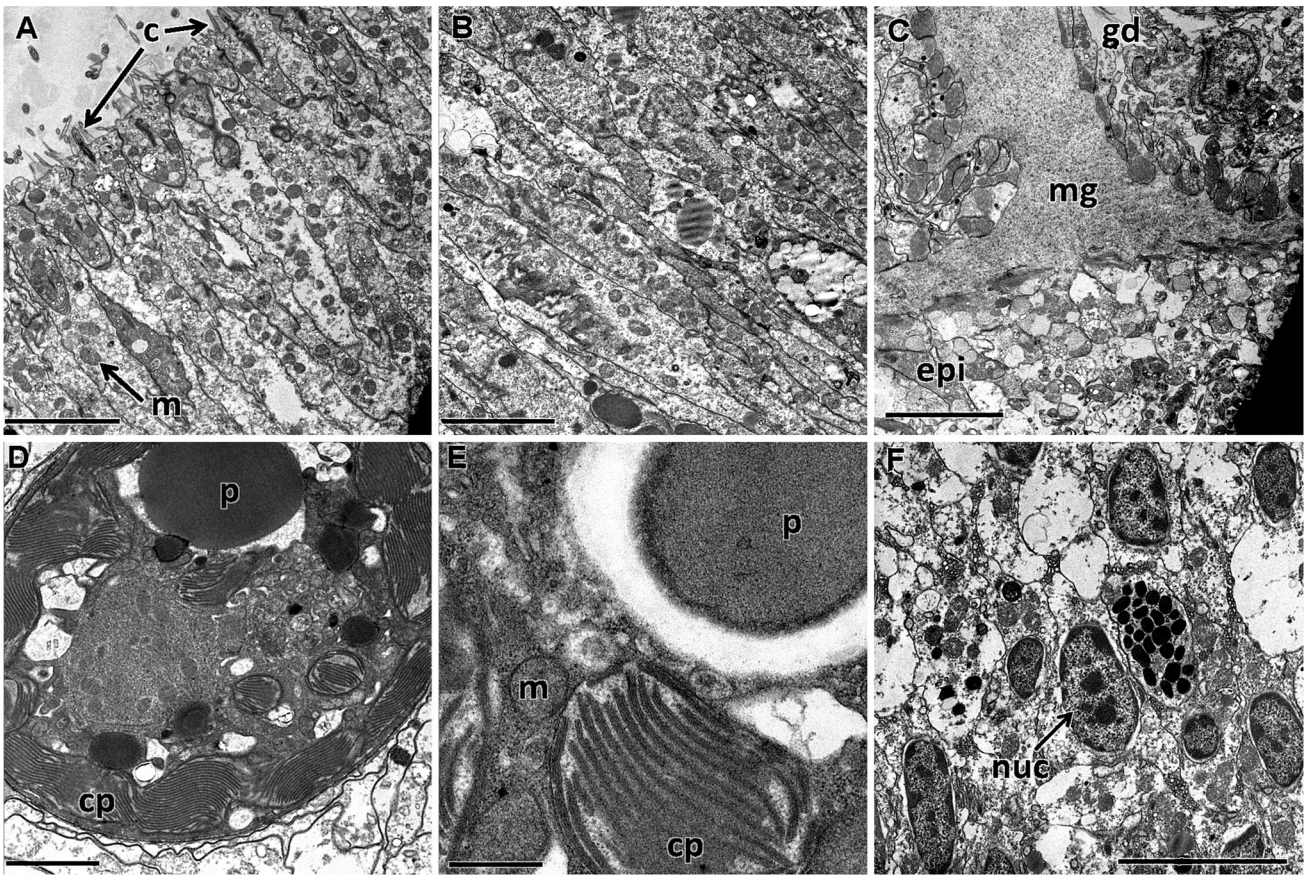

Fig. 3 Transmission electron microscopy of planula of *Stylophora pistillata* in the control treatment. **a** epidermal surface, indicating the presence of functional cilia (*c*), abundant mitochondria (*m*), and tightly adjoined epidermal cell types; *bar* indicates 5,000 nm. **b** deeper within the epidermal tissue; *bar* indicates 5,000 nm. **c** interface of epidermis (epi), gastrodermis (gd), and mesoglea (mg); *bar* indicates 5,000 nm. **d** micrograph indicating zooxanthella within gastrodermal cell. Notice the absence of a vacuolar space between the coral vacuolar membrane and the thecal plates/membrane of the zooxanthella. Chloroplasts (*cp*) and pyrenoid body (*p*) of the zooxanthella are indicated; *bar* represents 2,000 nm. **e** higher magnification of organellar structures of zooxanthella in the gastrodermal tissue of planula, indicating the presence of intact chloroplasts (*cp*), mitochondria (*m*) and pyrenoid body (*p*); *bar* indicates 2,000 nm. **f** area of gastrodermis between symbiotic gastrodermal cells and yolk, (*nuc*) indicated coral cell nucleus with intact double-layer nuclear membrane; *bar* indicates 500 nm

deformed planulae exposed in the light and dark were much lower: 315 ppb (1,280 nM) and 1.05 ppm (4,253 nM), respectively (Supplemental Fig. 2a, c). The corresponding EC_{20}s were just 7.14 ppb (29 nM) and 9.1 ppb (37 nM), respectively. PROBIT analysis for EC_{50}s for non-deformed planulae exposed in the light and dark were 535 ppb (2,172 nM) and 1.6 ppm (6,498 nM), respectively (Supplemental Fig. 4a, c). PROBIT analysis for EC_{20}s for non-deformed planulae exposed in the light and dark were 33 ppb (134 nM) and 14 ppb (56.8 nM), respectively (Supplemental Fig. 4a, c).

The number of DNA abasic sites increased approximately eight-fold across the BP-2 concentration gradient after an 8-h exposure, regardless of whether planulae were exposed in the light or dark (Fig. 6b, d). Non-linear regression estimation of DNA AP abasic sites EC_{20} and EC_{50} for BP-2 in the light are 52 ppt (0.211 nM) and 8.6 ppb (34.9 nM), respectively. Non-linear regression estimation of DNA AP abasic sites EC_{20} and EC_{50} for BP-2 in the dark are four ppt (0.016 nM) and 1.8 ppb (7.12 nM), respectively.

The percentage of dead coral cells increased dramatically with increasing concentrations of BP-2, but the LC_{50} was lower in the light (491 ppb; 1,993 nM) than in the dark (1.44 ppm; 5,847 nM) (Fig. 9b, d). The corresponding LC_{20}s in these two experiments were just 26 and 69 nM, respectively. PROBIT analysis for LC_{50}s for coral cells in both the light and the dark were 456 ppb (1,852 nM) and 1.027 ppm (4,1711 nM) (Supplemental Fig. 3).

Regression models used to estimate LC_{50}, EC_{20}, and EC_{50} of BP-2 after 24 h exposure had slightly more residual variation than in the short-term experiment, with

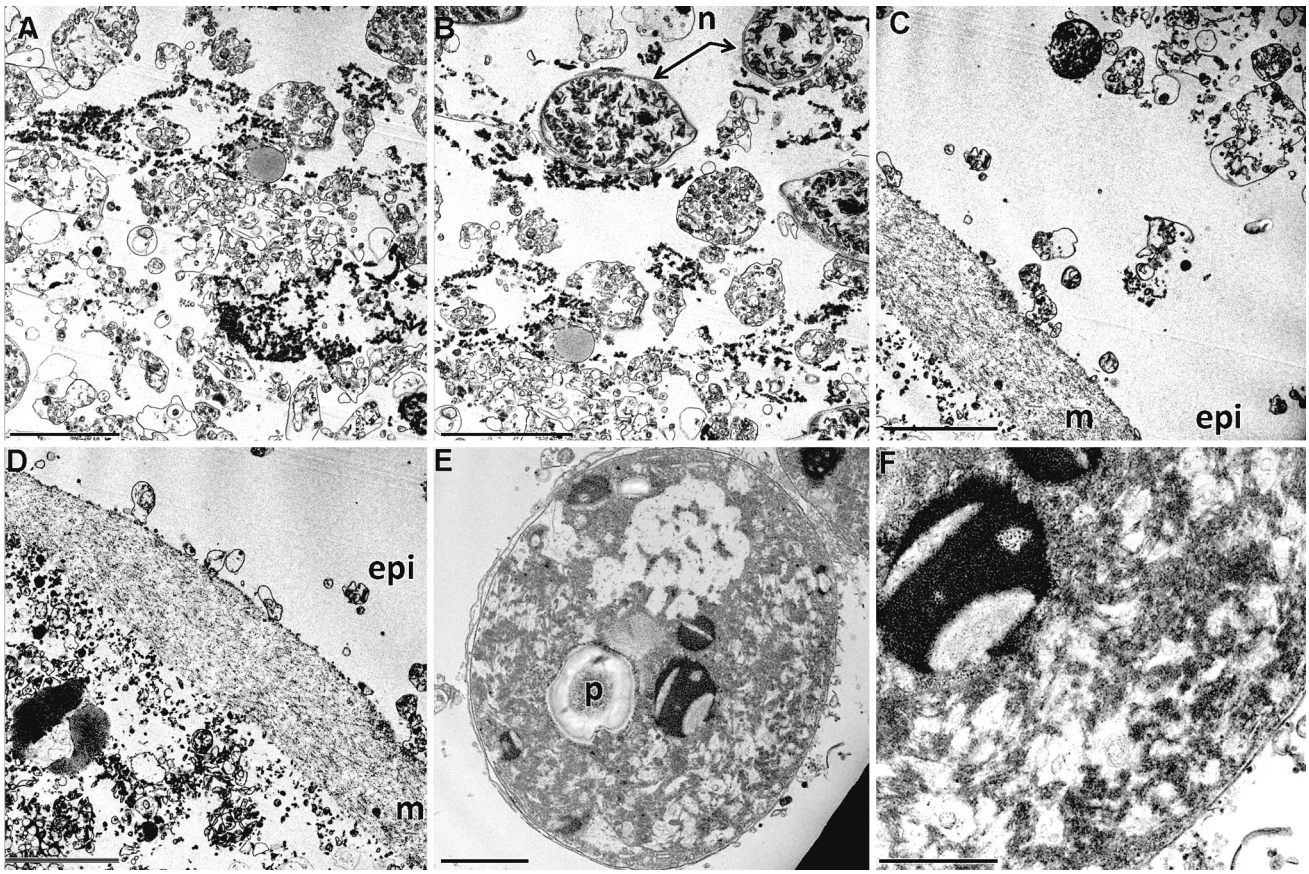

Fig. 4 Transmission electron microscopy of planula of *Stylophora pistillata* exposed to 246 ppm benzophenone-2 for 8 h in the light. **a** epidermal surface boundary zone, with disintegrated ciliated cells, epidermal granular cells, and goblet cells; *bar* indicates 5,000 nm. **b** cellular and tissue debris field of the epidermal layer of the planula. Cell membranes of nematocysts are intact, but the nematocytic bodies are disorganized; *bar* indicates 5,000 nm. **c** micrograph depicting the interface of epidermal (*epi*), mesoglea (*m*), and gastrodermal tissue boundaries; *bar* indicates 5,000 nm. **d** micrograph depicting the interface of epidermal (*epi*), mesoglea (*m*), and gastrodermal tissue boundaries; *bar* indicates 5,000 nm. **e** zooxanthella in gastrodermal tissue. Pyrenoid body (*p*) is disintegrated as well as complete disintigration of chloroplasts; *bar* indicates 2,000 nm. **f** magnification of massive inclusion body from **e**, typical of inclusion bodies found in all zooxanthellae at multiple concentrations of BP-2; *bar* indicates 1,000 nm

$R^2 = 0.72$–0.81. The LC_{50} for coral planulae exposed in the light was just 165 ppb (672 nM), while for planulae exposed in the dark, the LC_{50} was slightly higher: 508 ppb (2,063 nM) (Supplemental Fig. 1b, d). PROBIT analysis for LC_{50} of planula exposed to BP-2 in the light for 24 h was 223 ppb (905 nM) (Supplemental Fig. 5b). The bimodal behaviors of the graph (data) describing planulae exposed to the concentrations of BP-2 in the dark did not allow for a valid PROBIT analysis. A narrower array of concentrations that bracketed the transition range between the two modes of effect behavior may have allowed for PROBIT analysis. The EC_{50} for non-deformed planulae exposed to BP-2 in the light and dark were much lower: 19 ppb (77 nM) and 289 ppb (1,175 nM), respectively (Supplemental Fig. 2b, d). The corresponding EC_{20}s were very low: 246 ppt (1 nM) for planulae exposed in the light and 9.6 ppb (39 nM) for planulae exposed to BP-2 for 24 h in the dark. PROBIT analysis for EC_{50}s for non-deformed planulae exposed in the light and dark were 52 ppb (211 nM) and 428 ppb (1,738 nM), respectively (Supplemental Fig. 4b, d). PROBIT analysis for EC_{20}s for non-deformed planulae exposed in the light and dark were 4 ppb (16 nM) and 139 ppb (565 nM), respectively (Supplemental Fig. 4b, d).

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-2 concentrations, which makes cell mortality a potential indicator of reproductive failure. 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:

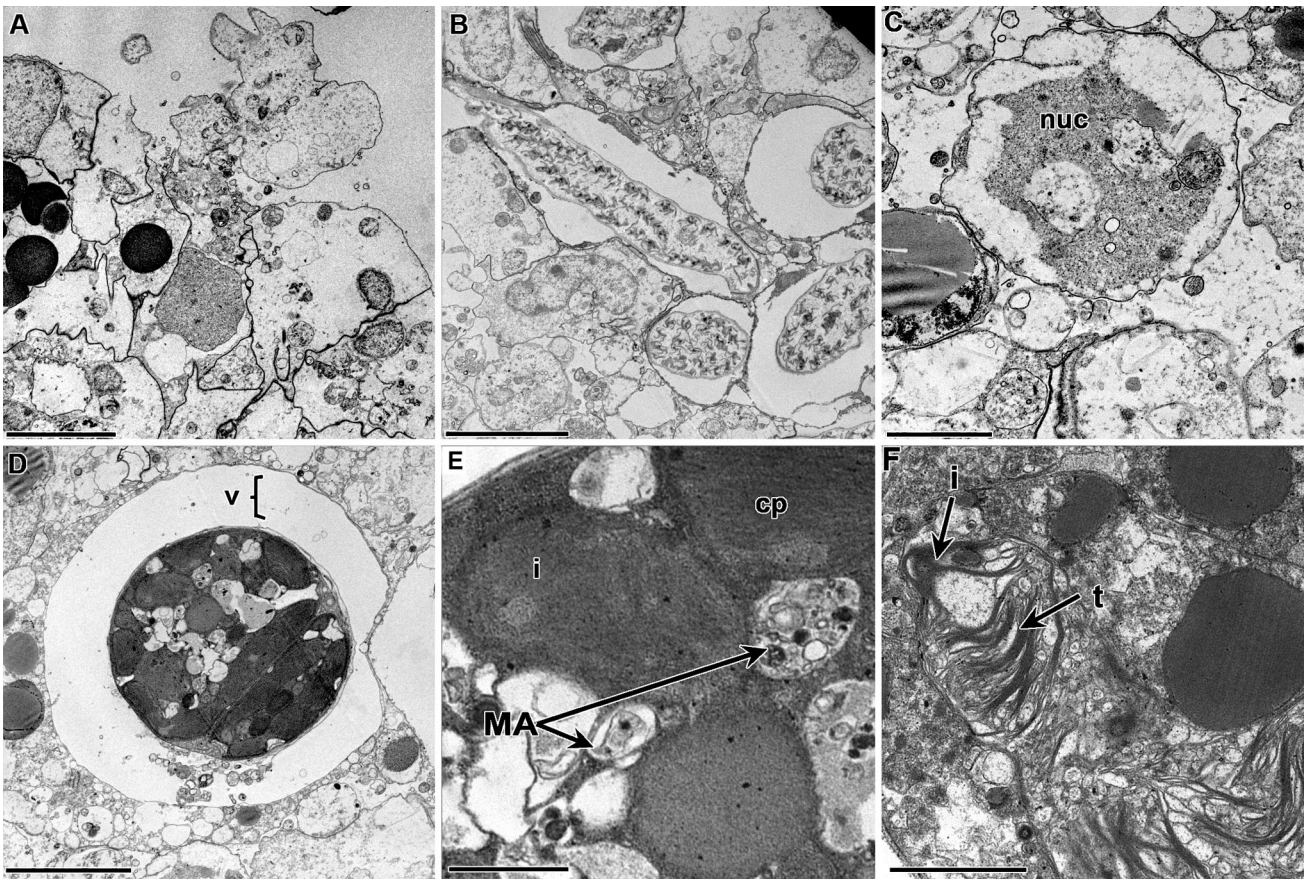

Fig. 5 Transmission electron microscopy of planula of *Stylophora pistillata* exposed to 246 ppm benzophenone-2 for 8 h in the dark. **a** surface of the epidermal layer, indicating loss of ciliated cells, presence of cellular necrosis, and disintegration of tissue coherency; *bar* indicates 5,000 nm. **b** deeper into the epidermal tissue layer with the presence of vacuolated nematocysts, vacuolated epidermal granular cells; *bar* indicates 5,000 nm. **c** necrotized cells in the gastrodermal tissue along the mesogleal boundary; *bar* indicates 2,000 nm (*nuc* nucleus). **d** symbiophagic vacuole surrounding zooxanthella in gastrodermal cell; (*v*) symbiophagic vacuolar space; *bar* indicates 5,000 nm. **e** chloroplasts in zooxanthella lacking thylakoid coherency and organelle decay; (*cp*) chloroplast; (*i*) dispersed thyalkoid membrane within chloroplast; (*MA*) = mitochondria undergoing autophagy; *bar* indicates 1,000 nm. **f** thylakoid membrane condensation in chloroplasts of zooxanthella; (*i*) biphasic separation of the trilayer thylakoid membrane; (*t*) early stages of thylakoid lamellae fusion; *bar* indicates 2,000 nm

In the light ($F_{2,21}$) = 2.62, $P < 0.0001$, $R^2 = 0.71$

$$\%\ \text{Mortality of planulae} = 15.2 - 1.49\,(\%\ \text{Mortality of cells}) + 0.0215\,(\%\ \text{Mortality of cells})^2$$

In the dark ($F_{2,21} = 27.0$, $P < 0.0001$, $R^2 = 0.72$).

$$\%\ \text{Mortality of planulae} = 11.7 - 1.04\,(\%\ \text{Mortality of cells}) + 0.161(\%\ \text{Mortality of cells})^2$$

Discussion

Benzophenone-2 induces different pathologies when planulae are exposed to either light or darkness, indicating that BP-2 is a phototoxicant. This is biologically and environmentally important, because corals will usually release brooded planulae at night (Gleason and Hofmann 2011). Planulae of spawning species (those that spawn eggs and sperm that are fertilized in the water column) are positively buoyant, residing at or near the surface of the ocean and may be obligated for two to four days to maintain a planktonic state before they are able to settle (Fadlallah 1983; Harii et al. 2007). Light levels on a clear sunny day in tropic latitudes can be as high as or higher than 2,000 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 exacerbate the phototoxicity. If exposed to BP-2 via common wastewater exposure routes, planulae will sequentially experience both forms of BP-2 induced

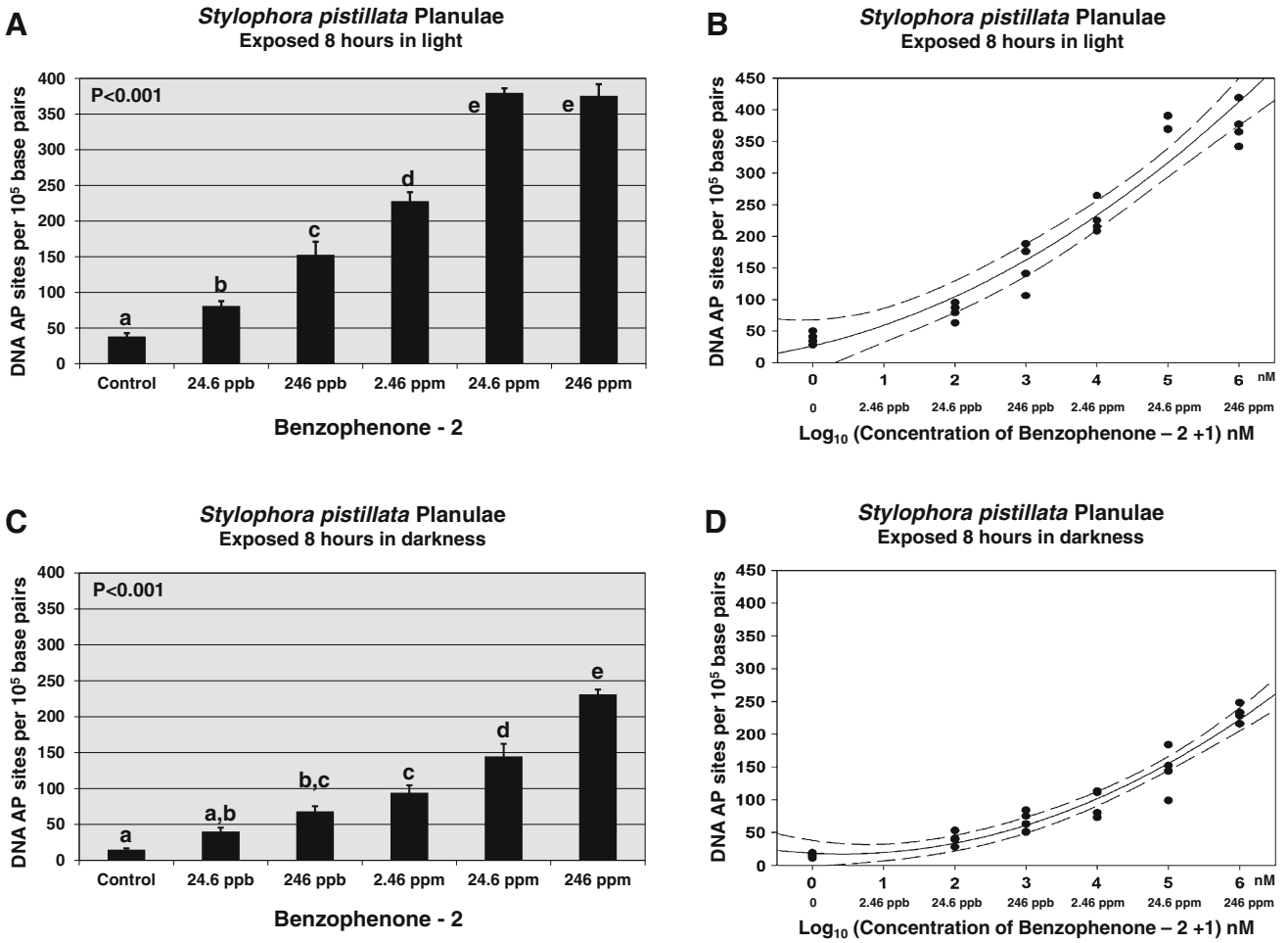

Fig. 6 Number of DNA apurinic/apyrimidinic lesions in planulae of *Stylophora pistillata* exposed to various concentrations of benzophenone-2 (BP-2). *Bars* show treatment means with whiskers representing ±1 standard error of the mean. Treatment means with *different superscript letters* differed significantly 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 *S. pistillata* exposed to concentrations of BP-2 for 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 *S. pistillata* exposed to concentrations of BP-2 for 8 h in the dark

pathologies. The danger is not only to near-shore reefs affected by shallow subsurface waters contaminated by septic fields or unmanaged cesspits, but also to off-shore reefs impacted by either piped wastewater effluent discharges or through ground-water seeps known to be contaminated with wastewater effluent or landfill leachates (Brooks et al. 2009; Futch et al. 2010; Pitarch et al. 2010).

Our data are consistent with the observation by Danovaro et al. (2008) that "sunscreen compounds" cause coral bleaching. In darkness, bleaching results from the symbiophagy of the symbiotic zooxanthellae; a process whereby the host cells "eats" the zooxanthellae (Downs et al. 2009). In the light, BP-2 causes damage directly to the zooxanthellae, independent of any host-regulated degradation mechanism. Based on the subcellular pathomorphology of the chloroplasts and thylakoids, BP-2 toxicity may result from the photo-oxidative stress to the molecular structures that form the membranes (Downs et al. 2013). Nesa et al. (2012) demonstrated that the algal symbionts of corals increase DNA damage to coral cells in coral planulae when exposed to sunlight, and is consistent with the Oxidative Theory of Coral Bleaching (Downs et al. 2002). If this is the case, then dark associated, BP-2-induced bleaching may reduce the exacerbated morbidity experienced by "bleached" planulae that would occur during times of daylight. Regardless of the toxicological mechanism, managing exposure of corals to BP-2 will be critical for managing coral reef resilience in the face of climate-change associated coral bleaching (Hughes and Tanner 2000; West and Salm 2003).

Consistent with the literature, BP-2 is a genotoxicant to corals (Cuquerella et al. 2012). The significant increase in

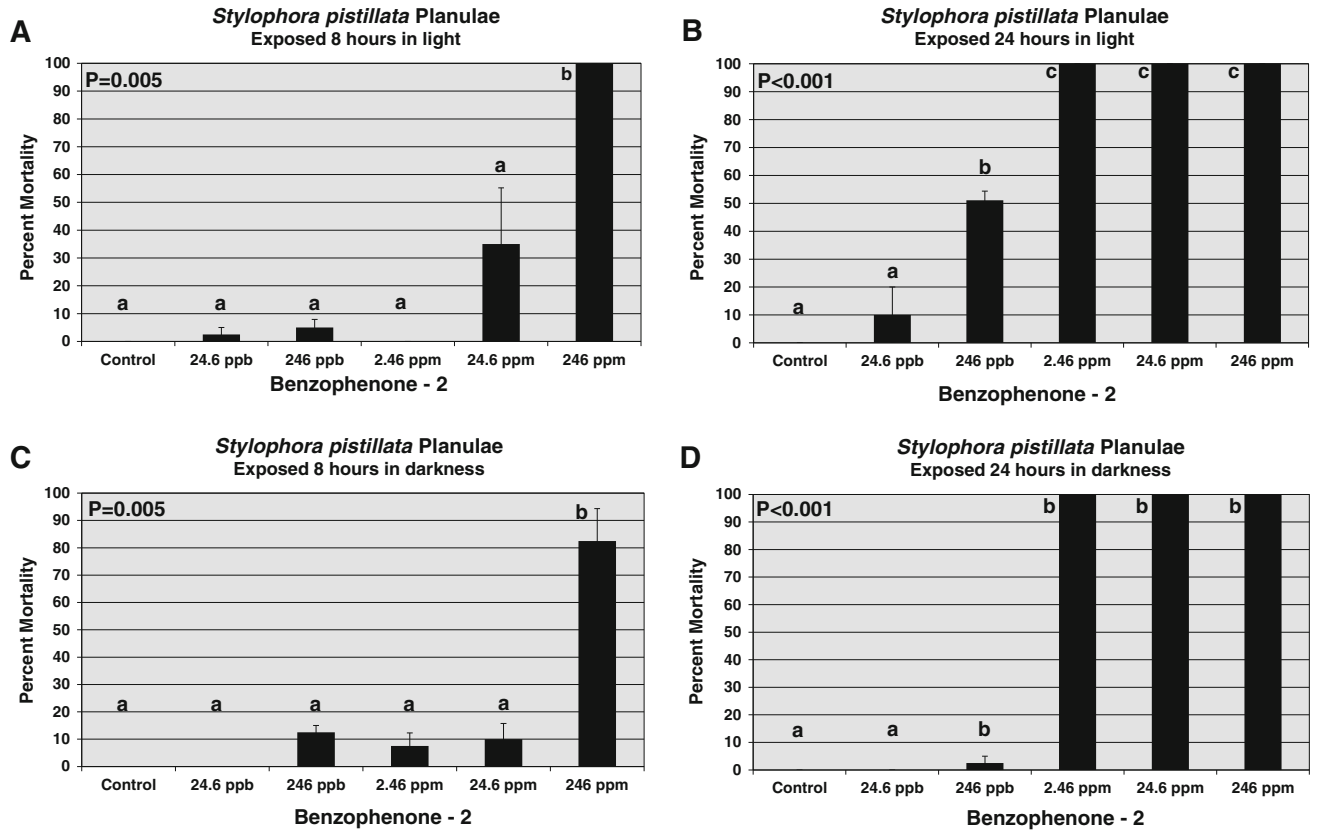

Fig. 7 Percent mortality of *Stylophora pistillata* planulae exposed to various concentrations of benzophenone-2 (BP-2). *Bars* show treatment means with whiskers representing ±1 standard error of the mean. Treatment means with *different superscript letters* differed significantly at $\alpha = 0.05$, 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

DNA AP sites of planulae exposed to BP-2 in the light versus in the dark is most likely the result of an oxidative-stress load resulting from photo-oxidative processes (e.g., formation of 8-oxo-dG; Sung and Demple 2006). Alternatively, increased DNA AP levels also may be in response to electrophilic alkylating agents resulting from the oxidative stress process or by metabolism of BP-2 into an alkylating agent (Fortini et al. 1996; Drablos et al. 2004). Accumulation of DNA damage in the larval state has implications for not only the success of coral recruitment and juvenile survival, but on reproductive effort and success (Anderson and Wild 1994; Depledge 1998). If planulae survive and develop into colonial adults after a morbid exposure to BP-2 that has assaulted genomic integrity, they may be unfit to meet the challenges of other stressor events, such as increased sea-surface temperature events. 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; in the adult coral, the somatic tissue directly gives rise to the germline during seasonal reproductive cycles. Damage to the genomic integrity of coral planulae may therefore have far-reaching adverse impacts on the fitness of both the gametes in adults stemming from affected planulae and their resulting offspring.

Autophagy was the dominant cellular response to exposure to BP-2 in darkness (Fig. 5a–d). Micro-autophagosomes were abundant in all cell types and larger vacuolated bodies of specific organelles were readily observed. Nuclei in all coral cell-types did not exhibit any of the classic signs of apoptosis, such as pyknosis or karyorrhexis of the nucleus (Krysko et al. 2008). Based on previous interpretations of cnidarian cell death (e.g., Kvitt et al. 2011; Paxton et al. 2013), one may argue that what is observed in this data set of micrographs is the result of apoptotic cells entering into a process termed "secondary necrosis" (e.g., Krysko et al. 2008). This argument would have validity if there was a marked absence of autophagic or phagocytosic bodies (e.g., autophagosomes); unfortunately, this is not the case. It may be that the best way to resolve these interpretations is to conduct experiments that incorporate timed-kinetics of cell death.

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

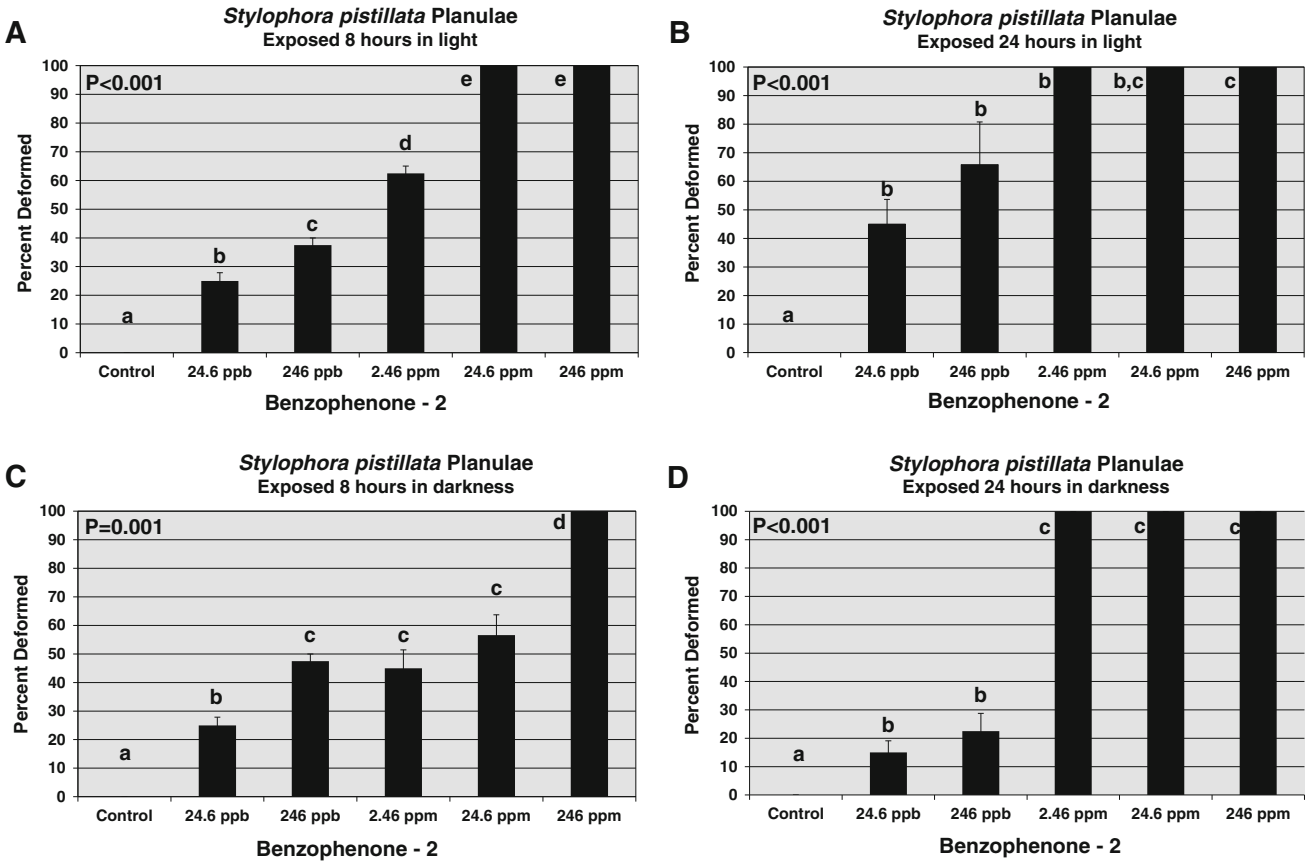

Fig. 8 Percentage deformed planulae of *Stylophora pistillata* exposed to various concentrations of benzophenone-2 (BP-2). *Bars* show treatment means with whiskers representing ±1 standard error of the mean. Treatment means with *different superscript letters* differed significantly at $\alpha = 0.05$, 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

being affected (Suter 1993; Suter and Mabrey 1994). *Stylophora*, used in this study, is indigenous to specific regions in the IndoPacific basins, and hence, may not be a valid representative for coral reef communities in Atlantic/Caribbean basins, or for specific coral reef habitats (e.g., high-energy zones such as barrier reefs or shorelines). The use of coral planulae in research studies is an incredibly difficult resource to obtain. It requires access to healthy coral colonies that are reproductively viable, obtaining the necessary collection and transport permits, and for many species, spawning may occur on a single night out of the entire year. We therefore developed an in vitro primary cell toxicity methodology using specific coral cell types that has been proposed as a surrogate for either planula or colonial polyp studies (Downs et al. 2010). Comparisons of LC_{50}s of coral cells in the light (456 ppb) and coral planula in the light for 8- and 24-h (120 ppm and 165 ppb, respectively) exhibit a similar response behavior. The increased sensitivity of in vitro cell models vs whole organism models is a common phenomenon and accepted principle (Blaauboe 2008; Gura 2008). Though 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.

Threshold values are units of measure that specify maximum permissible concentrations that an organism may be exposed to for a set length of time (e.g., allowable daily intake values for chemical contaminants in drinking water; Wennig 2000). In the proper context, threshold values can be used as a guide to set forth regulatory standards or help make mitigation decisions by resource managers during a natural resource damage event (Suter et al. 1987; Wennig 2000). LC_{50}, EC_{50}, EC_{20}, and NOECs are ecotoxicological values and were generated in this manuscript using different statistical methods to meet the expectations of various, and often conflicting, regulatory criteria and philosophies for the generation of these values

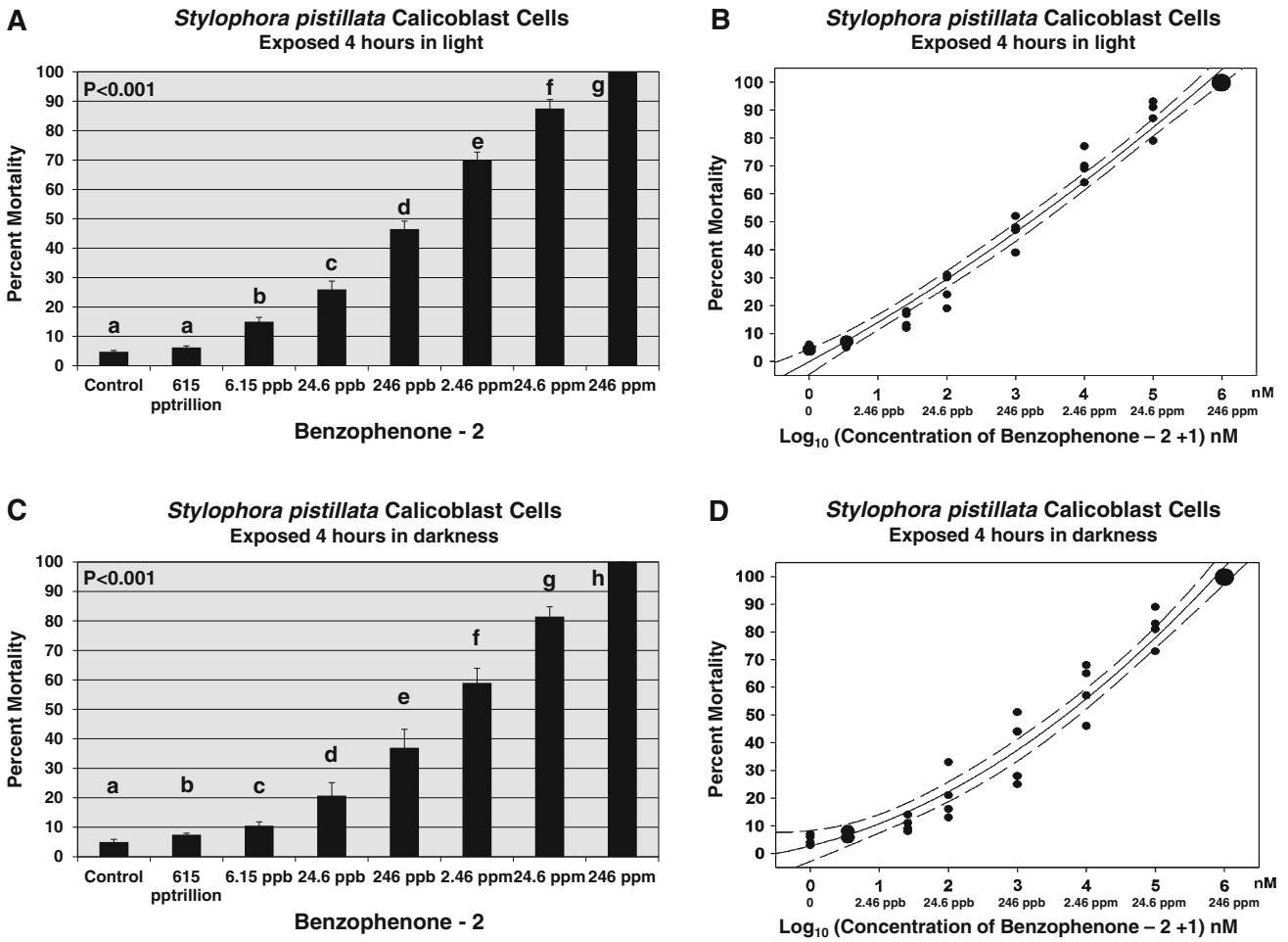

Fig. 9 Percent mortality of *Stylophora pistillata* calicoblast cells exposed to various concentrations of benzophenone-2 (BP-2). *Bars* show treatment means with whiskers representing ±1 standard error of the mean. Treatment means with *different superscript letters* differed significantly at $\alpha = 0.05$, based on one-way analysis of variance followed by a Holm–Sidak Method post hoc test. **a** calicoblast cells exposed for 4 h in the light. **b** Log-linear regression between coral cell mortality and concentrations of BP-2 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 light. **d** Log-linear regression between coral cell mortality and concentration of BP-2 for 4 h in the dark

(Laskowski 1995; Jager et al. 2006). These ecotoxicological values should in no way be confused as threshold values. Threshold or management-action values are often generated using specific formulations that may incorporate these ecotoxicological values (e.g., Suter 1993; Suter and Mabrey 1994; EC 2003). Resource managers, non-governmental organizations, and communities concerned about managing impacts of BP-2 should consult with professional ecotoxicologists and risk assessors for calculating appropriate threshold values. Development and application of BP-2 threshold-action levels for various coral reef habitats can be a powerful tool in managing the resilience of coral reefs at both the local level and globally.

In a forthcoming manuscript, we describe the induction of ossification of the planulae from exposure to benzophenone-3 (oxybenzone, BP-3), a structurally similar compound to BP-2. These two toxicological experiments were conducted concurrently, and the electron microscopy sample-processing was first conducted on BP-3 samples, then on BP-2 samples. Processing of the samples by the technicians was conducted without their knowledge of the samples' identity or their treatments. Unfortunately, by the time we realized the significance of the ossification in the BP-3 samples, the BP-2 samples were already processed for decalcification. For BP-3, there is some evidence in the scientific literature for endocrine disruption of osteogenesis and bone maintenance (Seidlova-Wuttke et al. 2004; Jarry et al. 2004). BP-2 should be tested using planulae from different coral species (i.e., planulae from brooding and broad-cast spawning species of coral) to determine if this compound induces a similar endocrine disrupting effect as oxybenzone.

Acknowledgments We thank Dr. Fuad Al-horani for his assistance, Ms. Maya Vizel for her assistance with the planula exposure challenges, Dr. Gideon Winters for assistance with Molecular Dynamics microplate fluorimeter, and the anonymous reviewers who greatly improved the quality of this manuscript. We also sincerely thank Dr. Sylvia Galloway and James H. Nicholson for their work on formatting the figures for publication.

Conflict of interest The authors can identify no potential conflicts of interest, neither financial or ethically, involved in the writing or publication of this manuscript.

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