

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/268154381>

# Establishment, Characterization, and Toxicological Application of Loggerhead Sea Turtle (*Caretta caretta*) Primary Skin Fibroblast Cell Cultures

ARTICLE *in* ENVIRONMENTAL SCIENCE AND TECHNOLOGY · NOVEMBER 2014

Impact Factor: 5.33 · DOI: 10.1021/es504182e · Source: PubMed

---

CITATION

1

READS

106

## 9 AUTHORS, INCLUDING:



[Sarah J. Webb](#)

Louisiana Universities Marine Consortium

1 PUBLICATION 1 CITATION

[SEE PROFILE](#)



[Benjamin M Higgins](#)

National Oceanic and Atmospheric Administra...

16 PUBLICATIONS 124 CITATIONS

[SEE PROFILE](#)



[Lauren Gollahon](#)

Texas Tech University

49 PUBLICATIONS 1,150 CITATIONS

[SEE PROFILE](#)



[Céline A J Godard-Codding](#)

Texas Tech University

20 PUBLICATIONS 221 CITATIONS

[SEE PROFILE](#)

## Establishment, Characterization, and Toxicological Application of Loggerhead Sea Turtle (*Caretta caretta*) Primary Skin Fibroblast Cell Cultures

Sarah J. Webb,<sup>†</sup> Gregory V. Zychowski,<sup>†</sup> Sandy W. Bauman,<sup>†</sup> Benjamin M. Higgins,<sup>‡</sup> Terje Raudsepp,<sup>§</sup> Lauren S. Gollahon,<sup>||</sup> Kimberly J. Wooten,<sup>†</sup> Jennifer M. Cole,<sup>†</sup> and Céline Godard-Codding\*,<sup>†</sup>

<sup>†</sup>The Institute of Environmental and Human Health, Department of Environmental Toxicology, Texas Tech University, 1207 Gilbert Drive, Lubbock, Texas 79409, United States

<sup>‡</sup>National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 4700 Avenue U, Galveston, Texas 77551, United States

<sup>§</sup>Department of Veterinary Integrative Biosciences, Texas A&M University, 4458 TAMU, College Station, Texas 77843, United States

<sup>||</sup>Department of Biological Sciences, 2901 Main, Texas Tech University, Lubbock, Texas 79409, United States

### Supporting Information

**ABSTRACT:** Pollution is a well-known threat to sea turtles but its impact is poorly understood. In vitro toxicity testing presents a promising avenue to assess and monitor the effects of environmental pollutants in these animals within the legal constraints of their endangered status. Reptilian cell cultures are rare and, in sea turtles, largely derived from animals affected by tumors. Here we describe the full characterization of primary skin fibroblast cell cultures derived from biopsies of multiple healthy loggerhead sea turtles (*Caretta caretta*), and the subsequent optimization of traditional in vitro toxicity assays to reptilian cells. Characterization included validating fibroblast cells by morphology and immunocytochemistry, and optimizing culture conditions by use of growth curve assays with a fractional factorial experimental design. Two cell viability assays, MTT and lactate dehydrogenase (LDH), and an assay measuring cytochrome P4501A (CYP1A) expression by quantitative PCR were optimized in the characterized cells. MTT and LDH assays confirmed cytotoxicity of perfluorooctanoic acid at 500  $\mu\text{M}$  following 72 and 96 h exposures while CYP1A induction was detected after 72 h exposure to 0.1–10  $\mu\text{M}$  benzo[a]pyrene. This research demonstrates the validity of in vitro toxicity testing in sea turtles and highlights the need to optimize mammalian assays to reptilian cells.



### INTRODUCTION

All seven extant species of sea turtle in the world are listed as vulnerable, endangered, critically endangered, or data-deficient (flatback sea turtle, *Natator depressus*) on the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species.<sup>1–3</sup> Anthropogenic risks to marine turtles are convoluted; in addition to habitat degradation, boat strikes, and entanglement in fishing gear, exposure to potentially toxic contaminants occurs both in the marine environment and on land.<sup>4–6</sup> Sea turtles are both migratory and long-lived, increasing chances for long-term exposure to multiple environmental contaminants. Very little is known about the effects of anthropogenic environmental contaminants on all reptile species compared to other taxa.<sup>7</sup> There is a critical need to reliably assess and monitor the effects of environmental pollutants on sea turtle populations within the legal constraints of their threatened or endangered status. Traditional invasive or lethal toxicology testing methods are very seldom suitable to endangered species research.<sup>8,9</sup> For this reason, toxicology

research in sea turtles so far has focused mainly on contaminant burden analyses in tissues,<sup>10–16</sup> with rare forays into animal dosing<sup>17,18</sup> or egg exposure studies.<sup>19,20</sup> Immortal cell lines were developed for virology in the green sea turtle (*Chelonia mydas*) almost exclusively from animals affected by fibropapillomatosis<sup>21–24</sup> or from embryos.<sup>25</sup> Pioneering in vitro toxicity testing in sea turtles used these cell lines to examine the effect of organochlorines on cytochrome P450 aromatase<sup>26</sup> and test metal sensitivity.<sup>27,28</sup> Primary skin fibroblasts and embryonic cultures were recently established and partially characterized in the hawksbill sea turtle (*Eretmochelys imbricata*) and olive ridley sea turtle (*Lepidochelys olivacea*).<sup>29–31</sup> Primary skin fibroblasts from one hawksbill sea turtle were subsequently evaluated for chromium toxicity<sup>32</sup> and

Received: August 26, 2014

Revised: October 27, 2014

Accepted: November 10, 2014

Published: November 10, 2014



a preliminary assessment of thermal effects on heat shock protein expression was conducted in embryonic cultures in the same species.<sup>32</sup> Additionally, leukocytes have been cultured from loggerhead turtles;<sup>12,33</sup> however, there are several major differences in the culture of blood cells versus adherent cells reported here. Blood cells typically require mitogen for proliferation and are short-lived. These leukocyte cultures were successfully used to test immune response following exposure to organochlorines.<sup>12</sup>

In vitro toxicology is well-established in medicine and pharmacology and relied upon heavily in industry in the case of drug trials. However, mammalian cells are used as the primary model system, and very little in vitro research has been conducted using reptilian cells. A series of recent studies<sup>26–28</sup> involved immortal cell lines derived from the same debilitated immature male green sea turtle afflicted with fibropapillomatosis.<sup>24</sup> While these studies provide important information and the cell lines are appropriate for virology-based research, extrapolation of toxicology data to the species level is limited by the following factors: (1) the diseased state of the source animal, (2) the fact that data derived from a single animal could reflect a phenotypic bias, and (3) the spontaneous (likely disease-induced) immortalization process, which may have caused cellular changes altering the capacity of the cell lines to accurately reflect in vivo processes. This last point is illustrated by the observed lack of cytochrome P450 19 (aromatase) induction, which could imply a missing induction pathway in the immortalized green turtle testis cell line.<sup>26</sup>

Here, we report on further advances regarding in vitro reptilian toxicology testing, including development and validation of novel methodologies for establishing and characterizing primary sea turtle cell cultures from skin biopsies, coupled with toxicological assessments. These primary cultures are well suited for in vitro research because they originated from skin biopsies of multiple young, healthy captive-reared loggerheads. The use of primary cell cultures circumvents the cellular alterations generally associated with immortalization, and deriving cell cultures from multiple individuals more accurately accounts for intraspecies variability.

The first objective of this study was to fully characterize the primary skin fibroblast cell cultures we established from the loggerhead sea turtle (*Caretta caretta*) by confirming the cell type and determining optimal growth conditions. Characterization is among the first and most critical steps in working with any cell culture,<sup>34</sup> yet it is often overlooked in cultures established from wildlife. Four factors known to influence cell growth (medium, serum, temperature, and plate coating/substrate) were tested by a single-factor manipulation or one-variable-at-a-time (OVAT) approach followed by a fractional factorial (FF) design.

The second objective of the study was to adapt standard in vitro toxicological assays to these unique reptilian primary cells. Two cytotoxicity assays, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH), and a biomarker assay for cytochrome P450 1A induction were selected. The MTT assay is based on the conversion of MTT salt to formazan in normal functioning mitochondria.<sup>35</sup> Lactate dehydrogenase is an enzyme normally present in the cytosol and periodically released through exocytosis. It is released in greater amounts in cells undergoing necrosis, apoptosis, or other mechanisms affecting the cell membrane than in healthy cells.<sup>36</sup> MTT and LDH assays were conducted following exposure to environmentally relevant

concentrations of perfluorooctanoic acid (PFOA), a common marine contaminant that has been detected in the serum of sea turtles<sup>37,38</sup> and is known to cause significant cytotoxicity in mammalian in vitro studies.<sup>39,40</sup> Quantitative real-time PCR (qPCR) was used to quantify the expression of cytochrome P4501A5 (CYP1A5) following exposure to benzo[a]pyrene (B[a]P), a prototypical polycyclic aromatic hydrocarbon (PAH) and common marine contaminant.<sup>41</sup> CYP1A5, a newly cloned reptilian gene belonging to the CYP1A subfamily,<sup>42</sup> is known to play a critical role in the metabolism, detoxification, and bioactivation of PAHs in mammals.<sup>43</sup> Polycyclic aromatic hydrocarbons have been detected in plasma or tissues of green, loggerhead, Kemp's ridley (*Lepidochelys kempii*), and leatherback (*Dermochelys coriacea*) sea turtles and in loggerhead eggs.<sup>10,15,16,44,45</sup>

## EXPERIMENTAL PROCEDURES

**Sampling Methods and Permits.** Cell cultures were derived from skin biopsies collected from healthy captive-reared animals held at the NOAA/NMFS Sea Turtle Facility in Galveston, Texas. All turtles sampled were between 2 and 4 years old. Biopsies were collected under the supervision of a veterinarian as 6–8 mm punches from the hind flipper and stored in cold supplemented RPMI 1640 medium (Mediatech) immediately upon collection [supplements were 1.0% penicillin–streptomycin (Mediatech) and 1.0% amphotericin B (Mediatech)]. Samples were shipped cold overnight to The Institute of Environmental and Human Health (TIEHH), Texas Tech University, Lubbock, TX.

All biopsies were collected under U.S. Fish and Wildlife Service Endangered Species Act Section 10a(1)a Scientific Research Permit TE-676379-4 and TE-676379-5 and Florida Fish and Wildlife Conservation Commission Marine Turtle Permit MTP-015. All research complied with all institutional animal care guidelines.

**Establishment of Primary Skin Fibroblast Cultures.** Upon arrival of biopsies at Texas Tech University, medium was exchanged for 10 mL of phosphate-buffered saline (PBS, HyClone) supplemented with 1.0% penicillin–streptomycin and 1.0% amphotericin B (Mediatech). After a 30 min 4 °C refrigeration, biopsies were briefly dipped in 70% ethanol three times for sterilization. The tissue was minced by scalpel in 30 °C medium and then transferred to a vented 25 cm<sup>2</sup> cell culture flask (Corning). Explants were arranged evenly on the growth surface, and flasks were maintained inverted without medium for 24 h in a Steri-Cult CO<sub>2</sub> incubator (Thermo Scientific) at 30 °C, 86% relative humidity (RH), and 5% CO<sub>2</sub>. Incubation then continued after addition of complete cell culture medium (shipping medium plus ~10% bovine calf serum, BCS) and reversion of flasks. Cells were harvested at or near cell confluence, counted, and transferred to either to 75 or 150 cm<sup>2</sup> vented flasks (Corning).

Three alternative methods of explantation were also tested in addition to the above standard laboratory explant procedure. These methods included (1) a scratched flask surface, (2) serum-coated flasks, and (3) varying timing of first medium change (see Supporting Information).

**Optimization of Primary Culture Growth Conditions.** Growth curve assays were used to test four critical factors contributing to cell growth: (1) medium type [RPMI 1640, MEM/EBSS (HyClone), or DMEM/F12 (HyClone)]; (2) growth surface coating [tissue culture (TC)-treated, TC-treated with gelatin (0.1%), TC-treated with collagen IV (2.5 µg/cm<sup>2</sup>,

Table 1. Combinations Tested by One-Variable-at-a-Time Approach<sup>a</sup>

trial	medium			temperature			serum			surface			
	RPMI 1640	MEM/EBSS	DMEM/F1	25 °C	30 °C	35 °C	5%	10%	15%	TC-treated	+ collagen	+ fibronectin	+ gelatin
1	×				×			×			×		
2		×			×			×			×		
3			×		×			×			×		
4	×			×				×			×		
5	×					×		×			×		
6	×				×			×			×		
7	×				×				×		×		
8	×				×			×				×	
9	×				×			×					×
10	×				×			×					×

<sup>a</sup>Optimal conditions were determined on the basis of growth as well as statistical differences in final cell counts at each time point ( $p \leq 0.05$ ).

Corning), or TC-treated with fibronectin (2.5  $\mu\text{g}/\text{cm}^2$ , Corning); (3) concentration of BCS in the medium (~5%, ~10%, or ~15%); and (4) incubation temperature (25, 30, or 35 °C), as seen in Table 1. For each of the 10 factorial combinations tested (Table 1), growth curves were performed  $\geq 3$  times with cells derived from different animals to incorporate potential biological variability. Initially, tests were performed via a single-factor manipulation or one-variable-at-a-time (OVAT) approach, in which only one variable was manipulated from the standard laboratory cell growth conditions for each test. For growth curve assays and for each factor tested, wells were seeded in triplicate with  $5 \times 10^4$  cells into seven 12-well plates (Greiner Bio One). Medium was replaced every 2 days. Cells were counted from one plate every 5 days until the end of the experiment at 35 days. Optimal conditions were determined on the basis of growth as well as statistical differences in final cell counts at each time point ( $p \leq 0.05$ ). The ten OVAT combinations were analyzed in JMP 8.0 statistical software by one-way analysis of variance (ANOVA). Significant differences and overall averages were used to select the two best conditions for each factor.

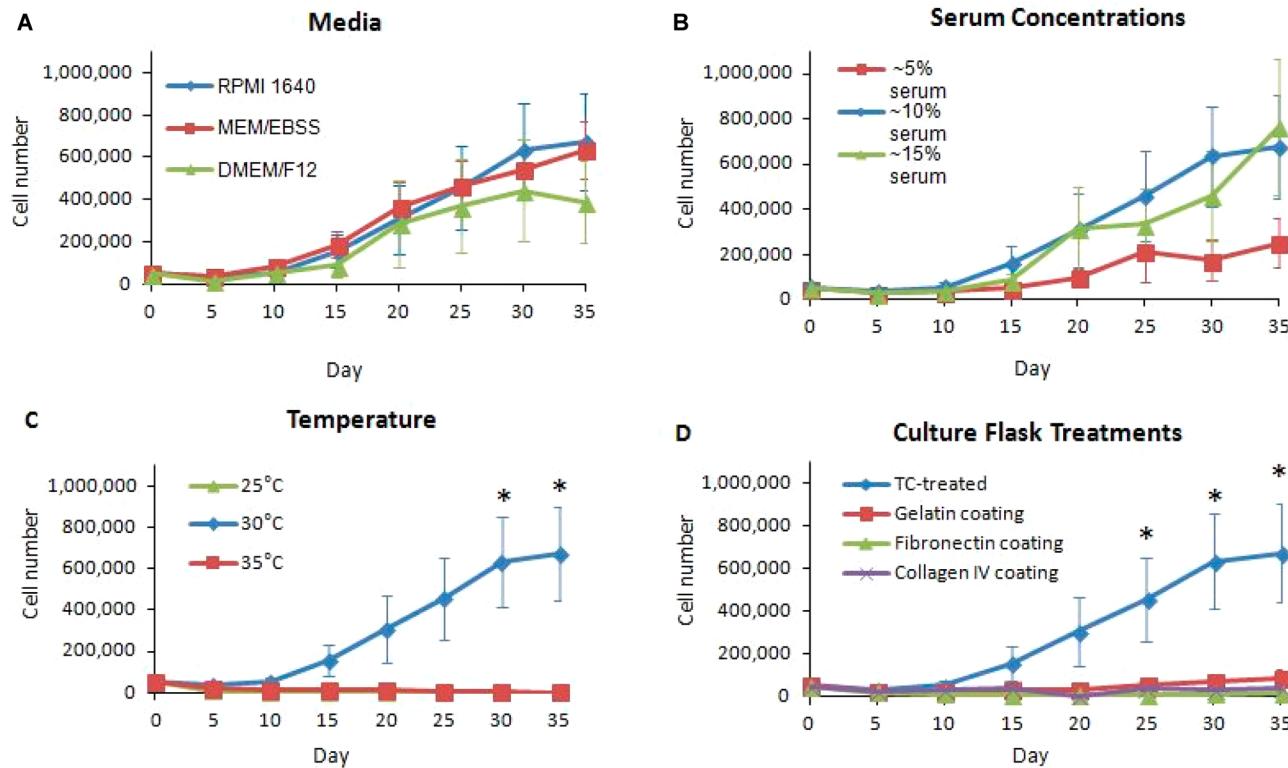
A fractional factorial (FF) design was then applied by selecting the best-performing options for each factor examined during the OVAT testing approach. The FF design incorporated two levels each for medium, serum, and coating/substrate but only one level for temperature since OVAT testing revealed that 30 °C greatly outperformed the other two temperatures. The design matrix was created by JMP 8.0 following a  $2_{III}^{3-1}$  format; that is, of the eight combinations possible for three factors at two levels each, the design captured four of these, described as the “first fraction,” with an acceptable compromise between confounding effects and number of combinations tested, such that the main effects could be detected.<sup>46</sup> Because three of the four combinations generated by the FF design had already been tested during OVAT testing, the only remaining combination in the matrix that required testing was that of MEM/EBSS with ~15% serum, with a 0.1% gelatin coating on TC-treated surface, and 30 °C incubation.

**Immunocytochemistry.** Cell identity was confirmed by immunocytochemistry (ICC) using an antibody against vimentin, a type III intermediate filament, known to be expressed in fibroblasts.<sup>47</sup> Approximately  $2.25 \times 10^5$  viable cells were seeded in each of 12 wells in a 48-well CellBind plate (Corning) with medium replaced every 2–3 days. At 90–95% confluence, cells were rinsed with room-temperature PBS and fixed in 100% ice-cold methanol (Fisher) for 20 min at –20 °C. Methanol was replaced by PBS and plates were kept at 4–5 °C

until ICC analysis. For ICC, PBS was replaced with phosphate-buffered saline containing 0.1% Tween 20 (PBST) (Genetex). Plates were rocked at room temperature for 10 min. PBS was aspirated, and a rabbit polyclonal antibody against vimentin (Abcam ab71144) was added at dilutions of 1:50 and 1:100 to three wells for each concentration. Cells were tested separately for the presence of cytokeratin 8 and 18, a type of intermediate filament characteristic of epithelial cells,<sup>47</sup> with the primary antibody NCL-L-5D3 (Novocastra) at a 1:100 dilution. Samples were screened for autofluorescence by use of a PBST-only treatment (no primary antibody). Plates were covered and rocked at 4 °C overnight, then rinsed with PBS three times and incubated with a rhodamine-conjugated, goat anti-rabbit IgG Red-X (Molecular Probes R6394) secondary antibody at a 1:250 dilution for 1 h. The nuclear counterstain 4',6-diamidino-2-phenylindole (DAPI, from VWR) was added to all wells after a PBST rinse for orientation purposes.

**Karyotyping.** Karyotyping followed the methods of Herbst et al.<sup>23</sup> Cells were cultured from a 2-year-old animal (sex undetermined) until they exhibited abundant mitotic activity, at which point demecolcine (Fisher) was added to arrest cells in metaphase. Cells were harvested and fixed with 3:1 methanol/glacial acetic acid. Following fixation, cells were stained with Giemsa and analyzed with a Zeiss AxioPlan2 microscope equipped with Ikaros image analysis software v5.2 (Meta-Systems GmbH).

**MTT Assays.** MTT assays were optimized for use with our characterized cell cultures. For each of six animals, cells were seeded with  $5 \times 10^4$  cells/well in TC-treated 96-well plates (Greiner Bio One) and maintained for 48 h at 30 °C to allow for cell attachment. Cells were then dosed in triplicate with PFOA (Sigma-Aldrich) at concentrations of 0.05, 0.5, 5.0, 50, and 500  $\mu\text{M}$  for either 72 or 96 h. This dose range encompasses concentrations found in seawater, prey items, and sea turtle tissues.<sup>37,38,48,49</sup> The stock solution of PFOA, which was serially diluted and used in dosing, was analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and was confirmed to be within 3.0% of the desired concentration (additional method details are available in Supporting Information). Two controls were used, one with vehicle only in medium (0.087% dimethyl sulfoxide, DMSO) and one with medium only. After the treatment period, medium containing PFOA was removed, 100  $\mu\text{L}$  of medium was added to each well, followed by 10  $\mu\text{L}$  of MTT (CalBiochem, prepared as 5 mg/mL in PBS, sterile-filtered). Incubation time for MTT metabolism was optimized for *C. caretta* cell cultures by testing 4, 12, and 24 h time points, and



**Figure 1.** Growth curve characterization assays for OVAT analysis in *C. caretta* primary skin fibroblasts: (A) medium tests, (B) serum concentration tests, (C) temperature variability analysis, and (D) coating/substrate analysis. Assays were performed in triplicate with cells from three or more animals. Bars represent standard error of the mean. Asterisks (\*) indicate statistical significance at  $p \leq 0.05$ .

24 h was found to be optimal to measure absorbance. Following 24 h MTT exposure, medium was removed from the plate and replaced with 100  $\mu$ L of DMSO to solubilize the formazan product. The plate was covered to eliminate light exposure, rocked for 30 min, and analyzed for absorbance at 570 and 650 nm on a BioTek Synergy 4 microplate reader with BioTek Gen5 software. Viability relative to the control was calculated, and one-way analysis of variance (ANOVA) followed by Tukey's posthoc analysis was performed with R (R Foundation for Statistical Computing, Vienna, Austria) to compare average viability for each dose.

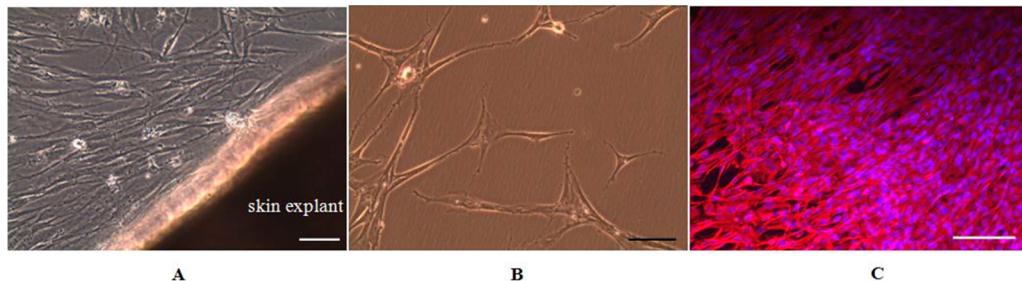
**Lactate Dehydrogenase Assays.** A lactate dehydrogenase cytotoxicity assay kit (Cayman Chemical 10008882) was utilized to quantify LDH concentration in cells from five individuals. Because this assay requires only medium, it was used in conjunction with MTT assays on cells dosed with PFOA as previously described. Medium was collected prior to the addition of MTT to wells. The tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) is metabolized to formazan in the presence of LDH, and cytotoxicity information is thus obtained by spectrophotometry. Absorbance was read on a BioTek Synergy 4 microplate reader at 490 nm. Standard curves were plotted as absorbance as a function of LDH concentrations and used to calculate LDH activity in each sample (microunits/milliliter). One-way ANOVA was used to determine statistical differences in LDH activity for cells at each dose and was calculated by use of the ISwR package in R ( $p \leq 0.05$ ) (R Foundation for Statistical Computing, Vienna, Austria), and a Tukey's test was used for posthoc analysis as necessary.

**Quantitative Real-Time Polymerase Chain Reaction.** For each of six animals,  $\sim 12 \times 10^6$  fibroblasts were divided

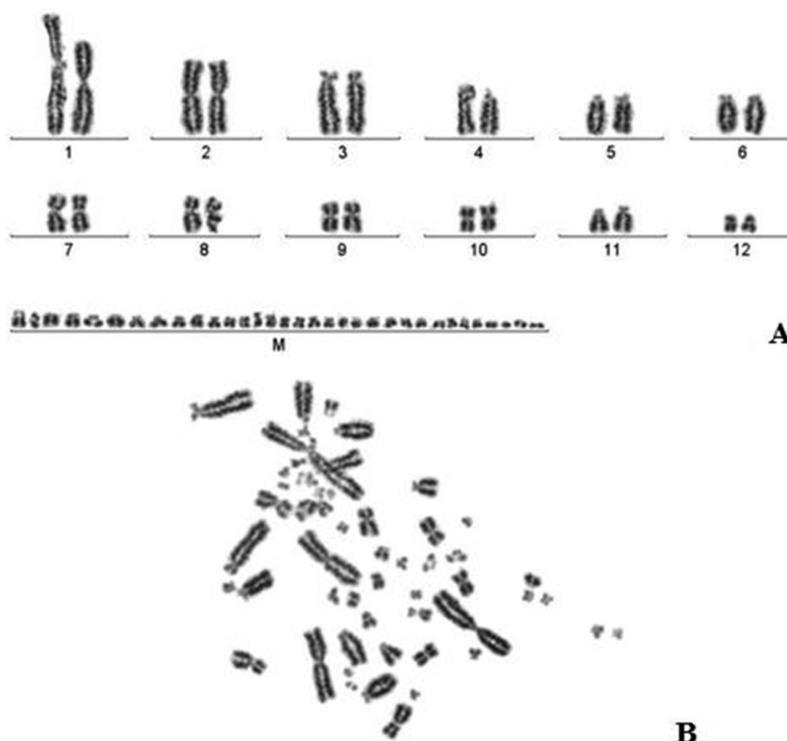
equally between five T-75 flasks and incubated in our optimized culture conditions ( $30^\circ\text{C}$ , 5.0%  $\text{CO}_2$ ) for 48 h to allow cell attachment. One control flask was exposed to 0.087% DMSO (carrier) only, and four treatment flasks were exposed to B[a]P at 0.01, 0.1, 1.0, or 10  $\mu\text{M}$  for 72 h, which represents what has been measured in sea turtle tissues.<sup>15,16,44</sup> The stock solution of B[a]P, which was used to make serial dilutions for dosing, was analyzed for accuracy by liquid chromatography with UV detection (LC-UV). Accuracy was confirmed within 3.5% of the target concentration (additional method details are available in Supporting Information). Total RNA was isolated by use of a NucleoSpinRNAII kit (Clontech, Foster City, CA) and integrity was evaluated by electrophoresis on 1% agarose gels in 1× 4-morpholinepropanesulfonic acid (MOPS) buffer.

Quantitative reverse-transcription polymerase chain reaction (qPCR) primers were designed for the loggerhead CYP1AS, actin, and 18S genes (Genbank accession numbers HQ293214, HQ828316.1, and HQ914786.1, respectively).<sup>42</sup> Fluorescence-based qRT-PCR was performed with the Rotor-Gene SYBR Green RT-PCR kit (Qiagen) by use of the liquid handling robot CAS-1200 and the Rotor-Gene 6000 (Corbett Research, Mortlake, New South Wales, Australia). Each sample was run in triplicate.

LinRegPCR software was used to generate a cycle threshold ( $C_t$ ) and amplification efficiencies for each sample.<sup>50</sup> 18S was selected as the best normalizing gene when compared to actin, on the basis of least amount of change in  $C_t$  values between tissue types or treatment groups and due to similar amplification efficiencies to CYP1AS (between 1.7 and 1.8).<sup>51</sup> qRT-PCR analysis of gene expression was carried out by the relative quantification  $\Delta\Delta C_t$  method ( $2^{-\Delta\Delta C_t}$ ).<sup>52</sup> For all treated groups, the expression ratio results were compared to control



**Figure 2.** *C. caretta* primary skin fibroblasts. (A) Fibroblasts proliferating from an explant (100 $\times$ , scale bar = 20  $\mu\text{m}$ ). (B) Fibroblasts in cell culture (100 $\times$ , scale bar = 10  $\mu\text{m}$ ). (C) Vimentin labeling by immunocytochemistry in *C. caretta* primary skin fibroblasts (100 $\times$ , scale bar = 50  $\mu\text{m}$ ). Vimentin is visualized by use of a rhodamine-conjugated, goat anti-rabbit IgG Red-X (red), and DAPI counterstained nuclei (blue). Images were captured by deconvolution fluorescence microscopy by use of a Hamamatsu Orca-ER high-speed camera on an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) at 100 $\times$ , and analyzed with Simple PCI software.



**Figure 3.** Analysis of chromosomes from 24 cells from a loggerhead sea turtle. (A) Karyotype of *C. caretta* primary fibroblasts showing  $2n = 56$ , with 24 macrochromosomes and 32 microchromosomes. (B) Metaphase spread from a *C. caretta* fibroblast.

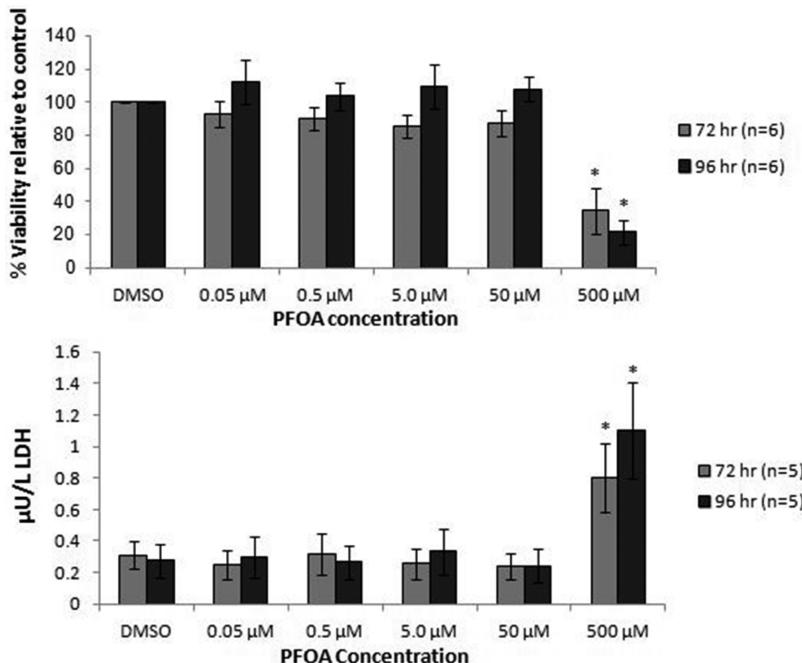
and tested for significance by a randomization test by use of the relative expression software tool REST.<sup>53</sup> A one-way ANOVA was also used to analyze  $\Delta\Delta C_t$  values for significance ( $p \leq 0.05$ ) between treatment groups. All statistical analysis was done using R (R Foundation for Statistical Computing, Vienna, Austria).

## RESULTS

**Optimization of Growth Conditions.** Our standard explantation method was the simplest and most cost- and time-effective of all four methods we tested and was selected for use thereafter. For each of these methods, time until confluence was recorded (Table S1, Supporting Information). Cell increases were observed over the course of 35 days for all media tested (Figure 1A). RPMI and MEM/EBSS yielded slightly higher averages than DMEM/F12 and were selected for the FF design. Serum concentrations of ~10% and ~15% were retained for the FF design on the basis of observed higher

yields (Figure 1B). Due to cell count results at or near 0 by day 35 for 25 and 35 °C, 30 °C was the single option retained for the FF design (Figure 1C). TC-treated polystyrene growth surface yielded the largest cell counts (Figure 1D) and was the most cost- and time-effective of all options tested. Statistically higher cell counts were obtained at days 25, 30, and 35 compared to the gelatin, fibronectin, and collagen treatments. Among options for additional coating, only gelatin produced an increase in cell numbers over the course of 35 days. Thus, the TC-treated surface without additional coating and the TC-treated surface with 0.1% gelatin coating were retained for the FF design (Table S2, Supporting Information). After statistical analyses, graphical representation of data, and cost considerations, the optimal combination of conditions was selected as RPMI 1640, 30 °C incubation, ~10% serum, and a TC-treated surface.

**Confirmation of Cell Type by Morphology and Immunocytochemistry.** Morphological characteristics of



**Figure 4.** PFOA cytotoxicity in *C. caretta* primary skin fibroblasts. (Top) MTT assays following 72 and 96 h exposure to PFOA with percent viability of treated cells normalized to performance of control (DMSO-treated) cells (mean  $\pm$  SEM,  $n = 6$ ). (Bottom) LDH assays following 72 and 96 h exposure to PFOA (mean  $\pm$  SEM,  $n = 5$ ). Asterisks (\*) indicate statistical significance at  $p \leq 0.05$ .

fibroblasts were consistent with what has been previously described in green sea turtle fibroblasts:<sup>24,54</sup> a spindle shape and varied branching (Figure 2A). Cells typically self-organized into densely packed, parallel arrangements upon reaching confluence (Figure 2B). ICC confirmed the presence of vimentin in the cells' cytoplasm (Figure 2C), providing further confirmation of fibroblast cell type. The anti-cytokeratin antibody used as the negative control, which is specific for epithelial cells, demonstrated no fluorescence after incubation. This experiment served to confirm the fibroblast cell traits as well as demonstrate the purity of cell culture.

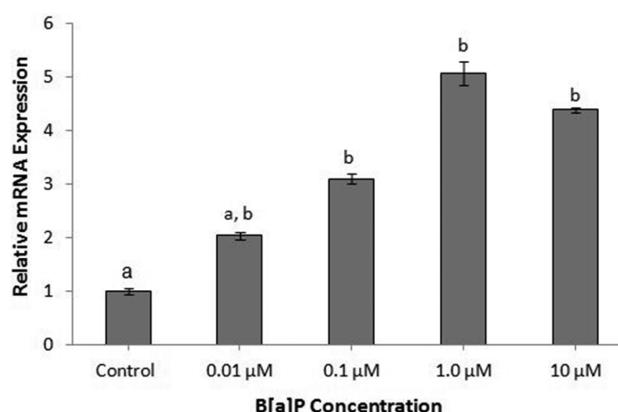
**Karyotyping.** Karyotyping showed that the diploid number of the loggerhead sea turtle is  $2n = 56$ , including 24 macrochromosomes (12 pairs) and 32 microchromosomes (16 pairs) and no sex chromosomes, which is standard for turtle species that exhibit temperature-dependent sex determination<sup>55</sup> (Figure 3). Chromosomes were counted in 24 cells from one individual, with 19 cells exhibiting  $2n = 56$  and six incomplete cells with either 55 or 54 chromosomes.

**Cytotoxicity Assays.** Significant cytotoxicity occurred in cells exposed to PFOA at the highest dose (500  $\mu\text{M}$ ) for both MTT assays ( $n = 6$ , 72 h  $p = 0.0001$ , 96 h  $p = 0.0001$ ) and LDH assays ( $n = 5$ , 72 h  $p = 0.032$ , 96 h  $p = 0.007$ ) (Figure 4).

**Biomarker Expression by qPCR.** CYP1A5 induction was detected in primary skin fibroblasts after exposure to 0.1, 1, and 10  $\mu\text{M}$  B[a]P ( $p$ -values of 0.002, 0.0001, and 0.0001, respectively) for 72 h (Figure 5). No differences were observed between treatments.

## DISCUSSION

Our optimal culture conditions were similar to the few previously described chelonian cell culture methods, with the biggest difference being the range of temperatures at which optimal proliferation occurred in other species. Previous literature suggests that temperatures at or slightly above the



**Figure 5.** Relative mRNA expression of CYP1A5 (normalized to the 18S gene) in loggerhead sea turtle primary skin fibroblasts exposed to B[a]P for 72 h (mean  $\pm$  SEM,  $n = 6$ ). Different letters indicate statistically different means within different treatment groups ( $p \leq 0.05$ ).

source animals' preference are suitable for reptilian cell culture work and that an optimal temperature would occur between approximately 23 and 37 °C for sea turtles.<sup>56–58</sup> Our findings indicated that 30 °C is the preferred growth temperature for primary loggerhead fibroblast sea turtle skin cultures and corresponds to the 28–30 °C rearing temperature at the NOAA Sea Turtle Facility. Immortal green sea turtle cells are reported to grow well at 25–30 °C, primary hawksbill and olive ridley sea turtle cells at 26 °C, and loggerhead sea turtle leukocytes at 30 °C, though proliferation was reported at a wide range of other temperatures.<sup>12,21,33,22–25,29,31</sup>

Reptilian cell cultures reported in the literature utilized cell medium designed for mammalian cells. Multiple options have been examined for sea turtle cell cultures, including medium 199, minimum essential medium (MEM), Dulbecco's modified

Eagle medium (DMEM), Leibovitz L-15, F-10 nutrient mix, F-12 nutrient mix, basal medium Eagle (BME), and RPMI 1640.<sup>21–25,29</sup> Our results show that more than one medium could be appropriate for loggerhead primary skin fibroblasts. This may be attributed to similarities in composition between RPMI 1640, MEM/EBSS, and DMEM/F12, although DMEM/F12 and RPMI 1640 have additional protein components that are absent in MEM.<sup>34</sup>

A concentration of 10% serum was optimal for previous sea turtle cell cultures.<sup>22–25,29</sup> Thus, our optimal serum concentrations of ~10% and ~15% were comparable and in agreement with existing literature. Results strongly suggested that loggerhead primary fibroblasts are serum-dependent, as medium without serum (data not shown) resulted in drastic declines in cell counts.

Proper attachment of cells to the growth surface is essential for normal cell physiology.<sup>59</sup> The polystyrene surface of cell culture dishes and flasks is a poor substrate for cell adhesion. Charged functional groups are added on the polystyrene to increase cell adhesion in TC-treated cell culture dishes and flasks.<sup>60</sup> Tissue adhesion to the flask surface seems to be a critical factor in initiating cell proliferation, and we investigated several modifications to the surface of tissue-culture-treated cell culture flasks known to increase the likelihood of tissue attachment for fibroblasts in other species.<sup>61</sup> These culture flask surface treatments had not been previously examined in sea turtle cell line studies. We observed that the TC-treated flask surfaces outperformed all other treatments tested.

Time until confluent monolayers are produced is markedly longer in our loggerhead cultures, at 45 days from establishment of tissue explants until first passage, compared to 7–12 days for immortal green sea turtle cultures and 14 days for primary hawksbill cultures.<sup>21,24,25,29</sup> The shorter time for green sea turtle is likely due to increased growth rates in immortal cells compared to primary cells. The difference in cell culture vessels between our study (15 cm<sup>2</sup> flasks) and the hawksbill study (35 mm, that is, 9.62 cm<sup>2</sup> dishes) likely accounts for differences in time until confluence, along with species specificity. Reptilian cultures have slower growth rates than mammalian cells, which tend to reach confluence in 3–7 days, as noted by both Stephenson,<sup>58</sup> for cells from the painted turtle, and Moore et al.,<sup>25</sup> for cells from the green sea turtle. This emphasizes the differences in physiology between mammalian and reptilian cells, highlighting the need for reptilian cell lines in particular to serve as a platform for toxicological studies.

Our karyotype results showed similar chromosome number to karyotypes reported for the green and hawksbill sea turtles. The first reported sea turtle karyotype for the green sea turtle described heterogametic chromosomes, with females 2n = 55 and males 2n = 56.<sup>62</sup> Subsequent studies disproved the heterogameticity of the species but confirmed a diploid number of 55 or 56 for the species.<sup>21,23,25,63</sup> Fukuda et al.<sup>29,30</sup> reported 2n = 56 in the hawksbill and olive ridley sea turtles, identical to our findings in the loggerhead (unknown sex).

Our data show that LDH and MTT cytotoxicity assays along with CYP1A gene expression quantitation are viable methods for investigating the impact of contaminant exposure to sea turtle cells. MTT assays have been used successfully in toxicity testing in immortal green sea turtle cell lines<sup>27,28</sup> and in loggerhead leukocytes,<sup>33</sup> although with a markedly shorter incubation time of 4 h. In our experience, MTT assay optimization for primary loggerhead fibroblasts required a longer incubation time of 24 h to allow the cells to fully

metabolize MTT and delineate between control and dosed cells, highlighting a critical need for species-specific and cell-specific optimizations of in vitro toxicity assays. Standard LDH methodologies worked well for our cultures. Gene expression of CYP1A5 via qPCR analysis used species-specific loggerhead sea turtle CYP1A5, 18S, and  $\beta$ -actin primers previously cloned in our laboratory.<sup>42</sup>

The lowest adverse effect level (LOAEL) for PFOA detected here in MTT and LDH assays, 500  $\mu$ M, is higher than levels reported in sea turtle tissues.<sup>37,38</sup> This does not necessarily indicate that PFOA levels found in tissues are not harmful, but merely that the levels are not high enough to directly cause cell death in fibroblasts in vitro within our tested exposure time (72 h). Research measuring perfluorocarbons in sea turtle tissues has indicated that levels are well above those known to cause adverse health effects in other species, including immune and neurobehavioral effects,<sup>37</sup> and further research is warranted to elucidate the exact impact of these contaminants to these animals.

We observed significant CYP1A5 induction in skin fibroblasts following a 72-h exposure to B[a]P starting at 0.1  $\mu$ M. Reports on the in vitro expression and inducibility of CYP1A in fibroblasts from terrestrial mammals are varied.<sup>64–67</sup> Interestingly, CYP1A induction in skin fibroblasts has been reported in marine mammals.<sup>8,68</sup> The establishment and characterization of primary skin cultures in the loggerhead open a much-needed avenue to further investigate the expression of CYP1A in these taxa.

Given the protected status of the loggerhead and other sea turtles, in vitro research is currently among the few viable options for studying these organisms with a high degree of experimental control. Tissue culture techniques provide much-needed tools for the investigation of toxicological and pathological threats in these and other protected species,<sup>69</sup> and the characterization of cell cultures is a critical first step in that process. To date, comparatively little research has been done toward the development of cell culture models for reptiles, leaving the state of knowledge far behind what is known for mammalian and even some invertebrate cells. The results from this research suggest that by minimally invasive sampling and use of the optimal conditions described, researchers now have the ability to use these primary cell cultures for a variety of purposes, including cytotoxicity testing. In vitro methods also have the advantage of reproducibility within and between laboratories, and we expect that our characterization and preliminary toxicological work will enable diverse research on a variety of threats to the loggerhead. Growth conditions such as percent serum in the medium, temperature, and confluence can have a major impact on the outcome of cytotoxicity assays.<sup>70–72</sup> Standard and consistent growth conditions are critical for replicable, reliable cytotoxicity data. This necessitates the establishment of standardized culture conditions to allow for accurate and repeatable assays among researchers. To the best of our knowledge, our research represents the most thorough characterization and optimization of any sea turtle primary adherent cell cultures. The establishment of these primary cell cultures from healthy animals provides new avenues of research in reptilian cell biology and toxicology. For example, the cultures we established can be used to obtain toxicity information on contaminants of current environmental concerns, such as crude oil exposure in marine turtles. Furthermore, our methodology is applicable to other protected species including the critically

endangered Kemp's ridley sea turtle (*L. kempii*), from which skin biopsies can be collected.

## ASSOCIATED CONTENT

### Supporting Information

Additional text with description of alternative methods for establishing fibroblast growth from tissue explants, explanation of cell count and population doubling level calculations, and methods for analyzing dosing stocks of B[*a*]P and PFOA via chromatography; two tables comparing cell number yields from different explant techniques and showing fractional factorial design. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## AUTHOR INFORMATION

### Corresponding Author

\*E-mail: [celine.godard@ttu.edu](mailto:celine.godard@ttu.edu); telephone: (806) 885-0337; fax: (806) 885-2132.

### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Funding

Funding was provided by the PADI Foundation and the Department of Interior/U.S. Geological Survey Deep Water Horizon Natural Resource Damage Assessment program.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We especially thank Dr. Greg Mayer, Dr. Jonathan Maul, Dr. Kamaleshwar Singh, Dr. Christopher Salice, and Dr. Michael Hooper for their valuable guidance throughout this research, the NOAA/NMFS sea turtle staff for their aid in collection of tissue, Jose-Luis Redondo and Janet Dertien for additional cell culture expertise, and Velvetee Finckbone and Zhi Pan for their expertise with immunocytochemistry.

## ABBREVIATIONS

CYP1A	cytochrome P4501A
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
LDH	lactate dehydrogenase
FF	fractional factorial
OVAT	one-variable-at-a-time
DMSO	dimethyl sulfoxide
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT)
BME	basal medium Eagle
DMEM	Dulbecco's modified Eagle medium
MEM	minimum essential medium
PAH	polycyclic aromatic hydrocarbon
PFOA	perfluorooctanoic acid
C <sub>t</sub>	cycle threshold
LC-UV	liquid chromatography with UV detection
ICC	immunocytochemistry
ANOVA	one-way analysis of variance
PAH	polycyclic aromatic hydrocarbon
qPCR	quantitative reverse-transcription polymerase chain reaction

## REFERENCES

- (1) Pritchard, P. C. H. Evolution, phylogeny, and current status. In *The Biology of Sea Turtles*; Lutz, P. L., Musick, J. A., Eds.; CRC Press: Boca Raton, FL, 1996; Vol. 1, pp 1–28.
- (2) Wallace, B. P.; DiMatteo, A. D.; Bolten, A. B.; Chaloupka, M. Y.; Hutchinson, B. J.; Abreu-Grobois, F. A.; Mortimer, J. A.; Seminoff, J. A.; Amorocho, D.; Bjorndal, K. A. Global conservation priorities for marine turtles. *PLoS One* **2011**, *6* (9), No. e24510.
- (3) IUCN, International Union for the Conservation of Nature Red List. <http://www.iucnredlist.org/>. Accessed January 14, 2014.
- (4) Gibbons, J. W.; Scott, D. E.; Ryan, T. J.; Buhlmann, K. A.; Tuberville, T. D.; Metts, B. S.; Greene, J. L.; Mills, T.; Leiden, Y.; Poppy, S. The Global decline of reptiles, déjà vu amphibians. *BioScience* **2000**, *50* (8), 653–666.
- (5) Hays, G. C. Sea turtles: A review of some key recent discoveries and remaining questions. *J. Exp. Mar. Biol. Ecol.* **2008**, *356* (1), 1–7.
- (6) Bolten, A. B.; Crowder, L. B.; Dodd, M. G.; MacPherson, S. L.; Musick, J. A.; Schroeder, B. A.; Witherington, B. E.; Long, K. J.; Snover, M. L. Quantifying multiple threats to endangered species: an example from loggerhead sea turtles. *Front. Ecol. Environ.* **2010**, *9* (5), 295–301.
- (7) Hopkins, W. A. Reptile toxicology: Challenges and opportunities on the last frontier in vertebrate ecotoxicology. *Environ. Toxicol. Chem.* **2000**, *19* (10), 2391–2393.
- (8) Godard, C. A.; Smolowitz, R. M.; Wilson, J. Y.; Payne, R. S.; Stegeman, J. J. Induction of cetacean cytochrome P4501A1 by β-naphthoflavone exposure of skin biopsy slices. *Toxicol. Sci.* **2004**, *80* (2), 268–275.
- (9) Godard, C.; Wise, S.; Kelly, R.; Goodale, B.; Kraus, S.; Romano, T.; O'Hara, T.; Wise, J., Sr. Benzo[*a*]pyrene cytotoxicity in right whale (*Eubalaena glacialis*) skin, testis and lung cell lines. *Mar. Environ. Res.* **2006**, *62*, S20–S24.
- (10) Alam, S.; Brim, M. Organochlorine, PCB, PAH, and metal concentrations in eggs of loggerhead sea turtles (*Caretta caretta*) from northwest Florida, USA. *J. Environ. Sci. Health, Part B* **2000**, *35* (6), 705–724.
- (11) Keller, J. M.; Kannan, K.; Taniyasu, S.; Yamashita, N.; Day, R. D.; Arendt, M. D.; Segars, A. L.; Kucklick, J. R. Perfluorinated compounds in the plasma of loggerhead and Kemp's ridley sea turtles from the southeastern coast of the United States. *Environ. Sci. Technol.* **2005**, *39* (23), 9101–9108.
- (12) Keller, J. M.; McClellan-Green, P. D.; Kucklick, J. R.; Keil, D. E.; Peden-Adams, M. M. Effects of organochlorine contaminants on loggerhead sea turtle immunity: comparison of a correlative field study and in vitro exposure experiments. *Environ. Health Perspect.* **2006**, *70*–76.
- (13) Day, R. D.; Christopher, S. J.; Becker, P. R.; Whitaker, D. W. Monitoring mercury in the loggerhead sea turtle, *Caretta caretta*. *Environ. Sci. Technol.* **2005**, *39* (2), 437–446.
- (14) Storelli, M.; Barone, G.; Marcotrigiano, G. Polychlorinated biphenyls and other chlorinated organic contaminants in the tissues of Mediterranean loggerhead turtle *Caretta caretta*. *Sci. Total Environ.* **2007**, *373* (2), 456–463.
- (15) Camacho, M.; Boada, L. D.; Orós, J.; Calabuig, P.; Zumbado, M.; Luzardo, O. P. Comparative study of polycyclic aromatic hydrocarbons (PAHs) in plasma of Eastern Atlantic juvenile and adult nesting loggerhead sea turtles (*Caretta caretta*). *Mar. Pollut. Bull.* **2012**, *64* (9), 1974–1980.
- (16) Camacho, M.; Luzardo, O. P.; Boada, L. D.; López Jurado, L. F.; Medina, M.; Zumbado, M.; Orós, J. Potential adverse health effects of persistent organic pollutants on sea turtles: evidences from a cross-sectional study on Cape Verde loggerhead sea turtles. *Sci. Total Environ.* **2013**, *458*, 283–289.
- (17) Lutz, P. L.; Lutcavage, M. *The effects of petroleum on sea turtles: Applicability to Kemp's ridley*; Texas A&M University Sea Grant Program: Galveston, TX, 1989.
- (18) Lutcavage, M.; Lutz, P.; Bossart, G.; Hudson, D. Physiologic and clinicopathologic effects of crude oil on loggerhead sea turtles. *Arch. Environ. Contam. Toxicol.* **1995**, *28* (4), 417–422.

- (19) Fritts, T.; McGehee, M. *Effects of petroleum on the development and survival of marine turtle embryos*; FWS-OBS-82/37; U.S. Fish and Wildlife Service Office of Biological Services, Washington, DC, 1982.
- (20) Podreka, S.; Georges, A.; Maher, B.; Limpus, C. J. The environmental contaminant DDE fails to influence the outcome of sexual differentiation in the marine turtle *Chelonia mydas*. *Environ. Health Perspect.* **1998**, *106* (4), 185.
- (21) Koment, R. W.; Haines, H. Characterization of a reptilian epithelioid skin cell line derived from the green sea turtle, *Chelonia mydas*. *In Vitro* **1982**, *18* (3), 227–232.
- (22) Mansell, J. L.; Jacobson, E. R.; Gaskin, J. M. Initiation and ultrastructure of a reptilian fibroblast cell line obtained from cutaneous fibropapillomas of the green turtle, *Chelonia mydas*. *In Vitro Cell. Dev. Biol.* **1989**, *25* (11), 1062–1064.
- (23) Herbst, L. H.; Sundberg, J. P.; Shultz, L. D.; Gray, B. A.; Klein, P. A. Tumorigenicity of green turtle fibropapilloma-derived fibroblast lines in immunodeficient mice. *Comp. Med.* **1998**, *48* (2), 162–167.
- (24) Lu, Y.; Nerurkar, V. R.; Aguirre, A. A.; Work, T. M.; Balazs, G. H.; Yanagihara, R. Establishment and characterization of 13 cell lines from a green turtle (*Chelonia mydas*) with fibropapillomas. *In Vitro Cell. Dev. Biol.: Anim.* **1999**, *35* (7), 389–393.
- (25) Moore, M. K.; Work, T. M.; Balazs, G. H.; Docherty, D. E. Preparation, cryopreservation, and growth of cells prepared from the green turtle (*Chelonia mydas*). *Methods Cell Sci.* **1997**, *19* (3), 161–168.
- (26) Keller, J. M.; McClellan-Green, P. Effects of organochlorine compounds on cytochrome P450 aromatase activity in an immortal sea turtle cell line. *Mar. Environ. Res.* **2004**, *58* (2), 347–351.
- (27) Tan, F.; Wang, M.; Wang, W.; Aguirre, A. A.; Lu, Y. Validation of an in vitro cytotoxicity test for four heavy metals using cell lines derived from a green sea turtle (*Chelonia mydas*). *Cell Biol. Toxicol.* **2010**, *26* (3), 255–263.
- (28) Wang, H.; Tong, J.; Bi, Y.; Wang, C.; Guo, L.; Lu, Y. Evaluation of mercury mediated in vitro cytotoxicity among cell lines established from green sea turtles. *Toxicol. In Vitro* **2013**, *27* (3), 1025–1030.
- (29) Fukuda, T.; Kurita, J.; Saito, T.; Yuasa, K.; Kurita, M.; Donai, K.; Nitto, H.; Soichi, M.; Nishimori, K.; Uchida, T. Efficient establishment of primary fibroblast cultures from the hawksbill sea turtle (*Eretmochelys imbricata*). *In Vitro Cell. Dev. Biol.: Anim.* **2012**, *48* (10), 660–665.
- (30) Fukuda, T.; Katayama, M.; Kinoshita, K.; Kasugai, T.; Okamoto, H.; Kobayashi, K.; Kurita, M.; Soichi, M.; Donai, K.; Uchida, T. Primary fibroblast cultures and karyotype analysis for the olive ridley sea turtle (*Lepidochelys olivacea*). *In Vitro Cell. Dev. Biol.: Anim.* **2014**, *50* (5), 381–383.
- (31) Takeshita, S.; Matsuda, N.; Kodama, S.; Suzuki, K.; Watanabe, M. In vitro thermal effects on embryonic cells of endangered hawksbill turtle *Eretmochelys imbricata*. *Zool. Sci.* **2013**, *30* (12), 1038–1043.
- (32) Wise, S. S.; Xie, H.; Fukuda, T.; Thompson, W. D.; Wise, J. P., Sr. Hexavalent chromium is cytotoxic and genotoxic to hawksbill sea turtle cells. *Toxicol. Appl. Pharmacol.* **2014**, *279*, 113–118.
- (33) Keller, J. M.; McClellan-Green, P. D.; Lee, A. M.; Arendt, M. D.; Maier, P. P.; Segars, A. L.; Whitaker, J. D.; Keil, D. E.; Peden-Adams, M. M. Mitogen-induced lymphocyte proliferation in loggerhead sea turtles: Comparison of methods and effects of gender, plasma testosterone concentration, and body condition on immunity. *Vet. Immunol. Immunopathol.* **2005**, *103* (3), 269–281.
- (34) Freshney, R. I. *Culture of Animal Cells: A Manual of Basic Technique*, 5th ed.; John Wiley & Sons, Inc.: New York, 2005.
- (35) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65* (1), 55–63.
- (36) Wolterbeek, H. T.; Van der Meer, A. J. Optimization, application, and interpretation of lactate dehydrogenase measurements in microwell determination of cell number and toxicity. *Assay Drug Dev. Technol.* **2005**, *3* (6), 675–682.
- (37) Keller, J. M.; Ngai, L.; McNeill, J. B.; Wood, L. D.; Stewart, K. R.; O'Connell, S. G.; Kucklick, J. R. Perfluoroalkyl contaminants in plasma of five sea turtle species: Comparisons in concentration and potential health risks. *Environ. Toxicol. Chem.* **2012**, *31* (6), 1223–1230.
- (38) O'Connell, S. G.; Arendt, M.; Segars, A.; Kimmel, T.; Braun-McNeill, J.; Avens, L.; Schroeder, B.; Ngai, L.; Kucklick, J. R.; Keller, J. M. Temporal and spatial trends of perfluorinated compounds in juvenile loggerhead sea turtles (*Caretta caretta*) along the east coast of the United States. *Environ. Sci. Technol.* **2010**, *44* (13), 5202–5209.
- (39) Freire, P. F.; Martin, J. M. P.; Herrero, O.; Peropadre, A.; de la Pena, E.; Hazen, M. J. In vitro assessment of the cytotoxic and mutagenic potential of perfluorooctanoic acid. *Toxicol. In Vitro* **2008**, *22* (5), 1228–1233.
- (40) Zhao, G.; Wang, J.; Wang, X.; Chen, S.; Zhao, Y.; Gu, F.; Xu, A.; Wu, L. Mutagenicity of PFOA in mammalian cells: Role of mitochondria-dependent reactive oxygen species. *Environ. Sci. Technol.* **2010**, *45* (4), 1638–1644.
- (41) Miller, K. P.; Ramos, K. S. Impact of cellular metabolism on the biological effects of benzo[a]pyrene and related hydrocarbons. *Drug Metab. Rev.* **2001**, *33* (1), 1–35.
- (42) Wiggins, S. *Identification and Characterization of Reptilian Cytochrome P450 1A: Sequencing, Expression, and Inducibility of CYP1As in Sea Turtles*; Texas Tech University, Lubbock, TX, 2011.
- (43) Conney, A. H. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: GHA Clowes Memorial Lecture. *Cancer Res.* **1982**, *42* (12), 4875–4917.
- (44) Hall, R. J.; Belisle, A. A.; Sileo, L. Residues of petroleum hydrocarbons in tissues of sea turtles exposed to the Ixtoc I oil spill. *J. Wildl. Dis.* **1983**, *19* (2), 106–109.
- (45) Godley, B.; Gaywood, M.; Law, R.; McCarthy, C.; McKenzie, C.; Patterson, I.; Penrose, R.; Reid, R.; Ross, H. Patterns of marine turtle mortality in British waters (1992–1996) with reference to tissue contaminant levels. *J. Mar. Biol. Assoc. U.K.* **1998**, *78* (03), 973–984.
- (46) Ryan, T. P. *Modern Experimental Design*. John Wiley & Sons, Inc.: Hoboken, New Jersey, 2007.
- (47) Moll, R.; Franke, W. W.; Schiller, D. L.; Geiger, B.; Krepler, R. The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. *Cell* **1982**, *31* (1), 11–24.
- (48) Yamashita, N.; Kannan, K.; Taniyasu, S.; Horii, Y.; Petrick, G.; Gamo, T. A global survey of perfluorinated acids in oceans. *Mar. Pollut. Bull.* **2005**, *51* (8), 658–668.
- (49) Gulkowska, A.; Jiang, Q.; So, M. K.; Taniyasu, S.; Lam, P. K.; Yamashita, N. Persistent perfluorinated acids in seafood collected from two cities of China. *Environ. Sci. Technol.* **2006**, *40* (12), 3736–3741.
- (50) Ramakers, C.; Ruijter, J. M.; Deprez, R. H. L.; Moorman, A. F. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* **2003**, *339* (1), 62–66.
- (51) McCurdy, R. D.; McGrath, J. J.; Mackay-Sim, A. Validation of the comparative quantification method of real-time PCR analysis and a cautionary tale of housekeeping gene selection. *Gene Ther. Mol. Biol.* **2008**, *12*, 15–24.
- (52) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta CT}$  method. *Methods* **2001**, *25* (4), 402–408.
- (53) Pfaffl, M. W.; Horgan, G. W.; Dempfle, L. Relative expression software tool (REST<sup>©</sup>) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **2002**, *30* (9), No. e36.
- (54) Work, T. M.; Dagenais, J.; Balazs, G. H.; Schumacher, J.; Lewis, T. D.; Leong, J.-A. C.; Casey, R. N.; Casey, J. W. In vitro biology of fibropapilloma-associated turtle herpesvirus and host cells in Hawaiian green turtles (*Chelonia mydas*). *J. Gen. Virol.* **2009**, *90* (8), 1943–1950.
- (55) Standora, E. A.; Spotila, J. R. Temperature dependent sex determination in sea turtles. *Copeia* **1985**, 711–722.
- (56) Wolf, K. Cold-blooded vertebrate cell and tissue culture. *Methods Enzymol.* **1979**, *58*, 466–477.
- (57) Wallace, B. P.; Jones, T. T. What makes marine turtles go: a review of metabolic rates and their consequences. *J. Exp. Mar. Biol. Ecol.* **2008**, *356* (1), 8–24.

- (58) Stephenson, N. G. Effects of temperature on reptilian and other cells. *J. Embryol. Exp. Morphol.* **1966**, *16* (3), 455–467.
- (59) Ruoslahti, E.; Pierschbacher, M. D. New perspectives in cell adhesion: RGD and integrins. *Science* **1987**, *238* (4826), 491–497.
- (60) Curtis, A.; Forrester, J.; McInnes, C.; Lawrie, F. Adhesion of cells to polystyrene surfaces. *J. Cell Biol.* **1983**, *97* (5), 1500–1506.
- (61) Grinnell, F.; Feld, M.; Minter, D. Fibroblast adhesion to fibrinogen and fibrin substrata: Requirement for cold-insoluble globulin (plasma fibronectin). *Cell* **1980**, *19* (2), 517–525.
- (62) Makino, S. The chromosomes of the sea turtle, *Chelonia japonica*, with evidence of female heterogamety. *Annot. Zool. Jpn.* **1952**, *25* (1), 250–257.
- (63) Bickham, J. W.; Bjorndal, K. A.; Haiduk, M. W.; Rainey, W. E. The karyotype and chromosomal banding patterns of the green turtle (*Chelonia mydas*). *Copeia* **1980**, 540–543.
- (64) Gradin, K.; Toftgård, R.; Poellinger, L.; Berghard, A. Repression of dioxin signal transduction in fibroblasts Identification of a putative repressor associated with Arnt. *J. Biol. Chem.* **1999**, *274* (19), 13511–13518.
- (65) Kim, P. M.; Deboni, U.; Wells, P. G. Peroxidase-dependent bioactivation and oxidation of DNA and protein in benzo[*a*]pyrene-initiated micronucleus formation. *Free Radical Biol. Med.* **1997**, *23* (4), 579–596.
- (66) Akintobi, A.; Villano, C.; White, L. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) exposure of normal human dermal fibroblasts results in AhR-dependent and-independent changes in gene expression. *Toxicol. Appl. Pharmacol.* **2007**, *220* (1), 9–17.
- (67) Henry, E. C.; Welle, S. L.; Gasiewicz, T. A. TCDD and a putative endogenous AhR ligand, ITE, elicit the same immediate changes in gene expression in mouse lung fibroblasts. *Toxicol. Sci.* **2009**, kfp285.
- (68) Fossi, M. C.; Casini, S.; Bucalossi, D.; Marsili, L. First detection of CYP1A1 and CYP2B induction in Mediterranean cetacean skin biopsies and cultured fibroblasts by Western blot analysis. *Mar. Environ. Res.* **2008**, *66* (1), 3–6.
- (69) Godard-Coddington, C. A.; Clark, R.; Fossi, M. C.; Marsili, L.; Maltese, S.; West, A. G.; Valenzuela, L.; Rowntree, V.; Polyak, I.; Cannon, J. C. Pacific Ocean-wide profile of CYP1A1 expression, stable carbon and nitrogen isotope ratios, and organic contaminant burden in sperm whale skin biopsies. *Environ. Health Perspect.* **2011**, *119* (3), 337.
- (70) Raz, A.; Fogler, W.; Fidler, I. The effects of experimental conditions on the expression of in vitro-mediated tumor cytotoxicity mediated by murine macrophages. *Cancer Immunol. Immunother.* **1979**, *7* (3), 157–163.
- (71) Hahn, G. M.; Shiu, E. C. Effect of pH and elevated temperatures on the cytotoxicity of some chemotherapeutic agents on Chinese hamster cells in vitro. *Cancer Res.* **1983**, *43* (12, Part 1), 5789–5791.
- (72) Hestermann, E. V.; Stegeman, J. J.; Hahn, M. E. Serum withdrawal leads to reduced aryl hydrocarbon receptor expression and loss of cytochrome P4501A inducibility in PLHC-1 cells. *Biochem. Pharmacol.* **2002**, *63* (8), 1405–1414.