Cytotoxicity of organic and inorganic compounds to primary cell cultures established from internal tissues of *Chelonia mydas*

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Abstract

Chemical contaminants have been found in the tissues of sea turtles from all over the world; however, very little is known about the effects. Recently, *in vitro* alternatives to live animal testing have been applied to sea turtles due to their ethical and practical benefits. While primary skin fibroblasts have been established for several species of sea turtle, cells from internal organs are lacking, though they may be more relevant due to the well documented accumulation of contaminants within internal tissues. This study established primary cell cultures from the small intestine, heart, liver, ovary and skin of green turtles (*Chelonia mydas*). Cells were exposed to ten contaminants typically found in sea turtles to examine potential variations in sensitivity among cells established from different organs. Differences between cells established from different animals were also examined, including a comparison of cells established from a turtle with fibropapillomatosis (FP) and healthy turtles. Loggerhead (*Caretta caretta*) primary skin cells were also included for species comparisons. Significant differences were found between the organ types, with liver and heart being the least sensitive, and skin being the most sensitive. Overall, variation between the organ types was low. Primary skin fibroblasts may be a suitable and representative cell type for *in vitro* turtle toxicology research, as it is relatively easy to obtain from healthy live animals. Skin cultures provide a more sensitive indication of effect, and could be used as an early warning of the potential effects of chemical contamination. Some species differences were found but no differences were found between cell cultures from an FP turtle and healthy turtles. When EC₅₀ values were compared to accumulation values from the literature, inorganic contaminants, such as Zn, Cd, Cr, Hg, and Cu were identified as posing a potential risk to sea turtle populations around the world.

Keywords

Organ variation; intra-individual variation; contaminants; cytotoxicity; green turtle;
loggerhead turtle

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1. Introduction

Some species of sea turtles forage in coastal areas that receive chemical contamination from urban, agricultural and industrial activities. In addition, sea turtles show strong fidelity to their foraging areas (Broderick et al. 2007), potentially resulting in long-term exposures to contaminants in these habitats. Accumulation of organic and inorganic contaminants has been reported in sea turtles from all over the world (See reviews by Pugh & Becker 2001, Keller 2003, D'Ilio et al. 2011, Cortés-Gómez et al. 2017). There is a growing body of evidence that suggests these contaminants can have negative effects in sea turtles (e.g. Keller et al. 2006, van de Merwe et al. 2010b, De Andrés et al. 2016). Sea turtles play integral roles in the ecosystem (Hamann et al. 2010) by influencing ecosystem dynamics (Yolanda & Karen 2002, Aragonés et al. 2006, Heithaus et al. 2007, Moran & Bjørndal 2007) and transporting nutrients and energy (Bouchard & Bjørndal 2000). Given that all species of sea turtle are listed by the International Union for Conservation of Nature (IUCN) as vulnerable (*Caretta caretta*, *Dermochelys coriacea*, *Lepidochelys olivacea*), endangered (*Chelonia mydas*), critically endangered (*Eretmochelys imbricata*, *Lepidochelys kempii*) or data deficient (*Natator depressus*) (IUCN 2018), it is critical to understand how contaminants found in turtles and their foraging grounds may be affecting turtle health.

There is unfortunately a severe knowledge gap in our understanding of how environmental contaminants can affect sea turtle health, because it is not practical or ethical to expose these large, endangered and long-lived marine animals to potentially toxic chemicals in controlled laboratory conditions. The recent establishment of sea turtle cells does, however, offer a promising avenue of investigation (Finlayson et al 2016), where cells can be used in *in vitro* toxicity bioassays to provide important species-specific information of the effects of chemicals in these animals at the molecular and cellular level. While cellular toxicity testing

does not provide a comprehensive understanding of toxicity to whole animals, it does offer the possibility of detecting the toxic molecular initiating event, the first step in chemical-induced toxicity (Allen et al. 2014). Primary skin fibroblasts have been established in recent years and used to assess the effects of a range of inorganic and organic contaminants (Webb et al. 2014, Wise et al. 2014, Young et al. 2015). Skin samples are easier to obtain than samples from internal organs, as they can be taken from live, healthy animals with minimal discomfort or stress to the animal. However, exposure through ingestion and accumulation in the internal organs (Keller 2013) suggest effects on internal organs may be more relevant. Recently, a primary lung fibroblast cell culture has been established for leatherback turtles (Speer et al. 2018). Only one study has established cell cultures from a range of internal organs of a green turtle (Lu et al. 1999). These cultures have since been used to examine cytotoxicity of several inorganic contaminants (Cd, Cr, Zn, Cu, Hg) (Tan et al. 2010, Wang et al. 2013). These studies found organ sensitivity varied between compounds, although some patterns were evident, such as liver cells often being the least sensitive. However, these cell cultures were obtained from a single turtle. For future toxicology studies, it is pertinent to investigate the variation between organ types, and whether a pattern of organ sensitivity can be identified across individuals regardless of the compound. Furthermore, the cell cultures used in previous studies were established from a turtle with fibropapillomatosis (FP). It has been suggested that cells established from a turtle with FP may be abnormal, missing for example important metabolic pathways (Keller & McClellan-Green 2004), or having irregular chromosome counts (Lu et al. 1999). Despite the limitations surrounding cell cultures from a diseased animal, turtles euthanized due to disease are often the only available source of fresh organ tissue for cell culture establishment for these endangered animals. It is therefore important to determine whether cells from animals with FP are acceptable to use in *in vitro* toxicological studies.

The first aim of this study was to examine differences in organ sensitivity by comparing the cytotoxicity of cells established from several different organ types to nine organic and seven inorganic compounds. The second aim was to compare cytotoxicity in cells established from green turtles with and without FP to identify any changes in sensitivity potentially attributed to the disease. Additionally, by including a loggerhead skin cell culture, differences in species sensitivity could be examined. Finally, effect concentrations were placed in an environmental context to prioritise populations at the highest risk

2. Methods

2.1 Sample collection

Skin samples were taken from a live green turtle basking in Hervey Bay and a live loggerhead turtle nesting at Mon Repos, Bundaberg, Queensland. The capture of all turtles was carried out under General Fisheries Permit number 139122. Organ samples were obtained from three green turtles that were either euthanized with Lethobarb injection for animal welfare reasons (including one with severe FP) at local wildlife hospitals, or that had recently died from physical injury (Table 1). Skin samples from live turtles (approx. 1 cm³) were taken using a scalpel following methods approved by Griffith University Animal Ethics Committee permit ENV/13/15/AEC. During necropsies of euthanized (performed by trained veterinarians) or deceased turtles, samples (approx. 1 cm³) were taken from selected tissues using a scalpel. All samples were kept in 5-10 mL of RPMI-1640 with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin (10,000 U/mL stock), 1% amphotericin B (250 µg/mL stock) and 0.5% gentamicin (50 mg/mL stock) and kept at 4°C until processing.

Table 1. Characteristics of sea turtles sampled to establish primary cell cultures

Species	Age	Sex	CCL (cm)	Location (activity)	Date	Tissue	Cell morphology	Cell code*
CM	SA	I	65	Hervey Bay (basking)	21/11/2015	skin	Fibroblast	GT06s- <i>p</i>
CM	J	I	47	Euthanized due to severe emaciation, heavy parasite load	11/04/2016	skin small intestine heart	Fibroblast Fibroblast Epithelial	GT08s- <i>p</i> GT08i- <i>p</i> GT08h- <i>p</i>
CM	J	F	50	Euthanized due to severe FP	6/07/2016	skin small intestine heart ovary liver	Fibroblast Fibroblast Epithelial Epithelial Epithelial	GT10s- <i>p</i> GT10i- <i>p</i> GT10h- <i>p</i> GT10o- <i>p</i> GT10l- <i>p</i>
CM	A	F	103.8	Died from physical injuries from a boat strike	10/10/2016	skin heart ovary	Fibroblast Fibroblast Epithelial	GT12s- <i>p</i> GT12i- <i>p</i> GT12o- <i>p</i>
CC	A	F	103.9	Mon Repos (nesting)	24/01/2016	skin	Fibroblast	LT05s- <i>p</i>

Abbreviations: CCL: curved carapace length; CM: *Chelonia mydas* (green turtle); CC: *Caretta caretta* (loggerhead turtle), A: adult; J: juvenile; SA: sub-adult; I: indeterminate; F: female; ND: no data. * The cell code identifies the species (first two letters), the individual (two numbers), the tissue (one letter), and is appended with the italicized letter “-p” to denote a primary cell.

2.2 Cell culture establishment

Methods for cell culture establishment and care have previously been described (Webb et al. 2014). Briefly, tissue samples were cut into 1-3 mm pieces in fresh RPMI-1640 media with FBS, 1% penicillin/streptomycin (10,000 U/mL stock), 1% amphotericin B (250 µg/mL stock). Tissue pieces were then transferred into a 25 cm² culture flask arranged evenly on the bottom of the flask. Media was removed, and flasks were inverted and incubated at 30°C, 5% CO₂. Following 24 h incubation, the flasks were reoriented, replenished with 5 mL of media

(described above) and returned to the incubator. Media was changed every 4 d. Tissue pieces were carefully detached and removed once flasks were ~70% confluent. The cells were washed twice with phosphate-buffered saline (PBS), detached using 0.25% trypsin, and transferred to a 75 cm² culture flask. Following this, cells were passaged each time they became ~70% confluent. Cells were cryopreserved at a concentration of 1×10^6 cells/mL in the RPMI media described above supplemented with 10% dimethyl sulfoxide (DMSO) as a preservative until ready to be used.

2.3 Cytotoxicity analysis

Primary cells from skin and internal tissues of a green turtle with FP (skin GT10s-*p*; liver, GT10l-*p*; small intestine, GT10i-*p*; ovary, GT10o-*p*; heart, GT10h-*p*), and two turtles without FP (heart, GT08h-*p*, GT12h-*p*; skin, GT08s-*p*, GT12s-*p*; small intestine, GT08i-*p*; ovary, GT12o-*p*) were established for the purposes of this study (Table 1). Skin was selected as this is the most common tissue type for sea turtle cell establishment. Liver was selected as it is the primary filter organ, small intestine was selected as turtles are mostly exposed to contaminants through ingestion. Heart and ovary have been shown to accumulate relatively high concentrations of particular contaminants compared to other tissue types (Sakai et al. 2000b, Lam et al. 2004). GT06s-*p* and LT05s-*p*, cell cultures were also included for species comparisons, as they were found to have low between trial variability in preliminary trials. Nine organic and seven inorganic compounds were screened for cytotoxicity using GT06s-*p*: N,N-diethyl-meta-toluamide (DEET, Sigma-Aldrich, analytical standard 97.6%), atrazine (Sigma-Aldrich, analytical standard 99.1%), metolachlor (Sigma-Aldrich, analytical standard 97.6%), phenanthrene (Sigma-Aldrich, ≥99.5%), diuron (Sigma-Aldrich, ≥98%), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153, AccuStandard, 99.9%), 4,4'-dichlorodiphenyldichloroethylene (4,4'-DDE, Sigma-Aldrich, 99%), dieldrin, Aroclor 1254

(AccuStandard, tech mix), copper (as $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, Chem-Supply, >99%), cobalt (as $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, Sigma-Aldrich, $\geq 98\%$), mercury (as HgCl_2 , Sigma-Aldrich), cadmium (as $\text{Cd}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$, Sigma-Aldrich, 98%), zinc (as ZnCl_2 , Sigma-Aldrich, 98%), lead (as $\text{Pb}(\text{NO}_3)_2$, Sigma-Aldrich, 99%) and chromium (as Na_2CrO_4 , Sigma-Aldrich, 98%). These contaminants were selected due to their ubiquitousness in aquatic systems, widespread use, or accumulation in turtles.

A resazurin assay was used to measure cell viability in response to contaminant exposure. Cells were seeded into a 96-well plate at 30,000 cells per well (in 100 μL of media) and incubated for 24 h. The resazurin assay is highly comparable with other cytotoxicity assays (Abdul Majeed et al. 2013, van Tonder et al. 2015, Taju et al. 2017) and may even be more sensitive (Hamid et al. 2004, Bigl et al. 2007). In a separate 96-well plate, contaminants were serially diluted in test media. Each concentration was tested in duplicate. Organics were prepared in methanol (MeOH), while the inorganics were prepared in ultrapure water (18.2 $\text{M}\Omega\cdot\text{cm}$). Both MeOH and water were used as negative controls and 1% Triton X-100 was used as a positive control. Final concentrations of the carrier solvents were 10%, as these concentrations had no effect on cell viability. Cell lines did not differ in their response to Triton X-100; all cells were killed in every case (as determined by fluorescence units equal to plate background levels). Following the initial 24 h incubation period, the chemicals (and controls) were added to the cells. Following 24 h of exposure, the media in each well was replaced with 80 μL of fresh media and 20 μL of 0.15 mg/mL resazurin dye in PBS, and incubated for a further 24 h. The fluorescence in each well was measured at $\lambda_{\text{ex}} = 544 \text{ nm}$ and $\lambda_{\text{em}} = 590 \text{ nm}$ using a fluorescence plate reader (FLUOstar Omega, BMG Labtech). Each compound was tested on three separate occasions. All cells were used between passage six

and 15 throughout the experiment, with the exception of GT06s-*p* which was used up to passage 22.

2.4 Data analysis

The percent of non-viable cells in each contaminant concentration was calculated using the equation: non-viable cells (%) = $(x - C_N)/(C_P - C_N) \times 100$, where x is the mean fluorescence of the sample duplicates, C_N is the average fluorescence of the solvent control, and C_P is the average fluorescence of the positive control. Concentrations of the inorganic compounds were presented as the metal ion equivalents. The non-viable cells (%) values were plotted against the log concentration of contaminants, and the half-maximal effect concentration (EC_{50}) values were calculated in GraphPad Prism 5 using the Hill slope equation.

To analyse for differences in EC_{50} values among organ types (for green turtle cell cultures only), nested ANOVAs were carried out with individual turtle nested within organ. LSD post-hoc analyses were carried out to examine differences in EC_{50} values among organ types when the ANOVA was significant. Mean EC_{50} values for each organ type were ranked from 1 (most sensitive) to 5 (least sensitive). The ranks were analysed using an ANOVA and LSD for differences in the order of organ sensitivity. Differences between species for each compound were also investigated using one-way ANOVAs on EC_{50} values. All analyses were performed in SPSS version 22 (IBM, USA).

Data for all GT10 cell cultures and GT06s-*p* for DEET and Aroclor 1254 were excluded from all analyses due to experimental error. Cell cultures with a relative standard deviation (RSD; $SD/mean \times 100$) between assay runs of $> 70\%$ for a given compound were also excluded from analysis for that compound (see Results section).

2.5 Chemical risk assessment

A screening level risk assessment was carried out using contaminant accumulation concentrations from the literature. For the purposes of comparisons, all values were converted to $\mu\text{g/g}$ wet weight (ww). For blood and plasma, conversions from mass per volume assume that blood is the same density as water, as the density of turtle blood and plasma are unknown and cannot be measured accurately without the mechanical oscillator technique (Kenner 1989). Concentrations in blood given as dry weight (dw) were converted to ww using a mean water content of 80.4% found by Guirlet et al. (2008). Organ concentrations given as $\mu\text{g/g}$ dw, have been converted to ww using ratios presented by Anan et al. (2001), ww:dw ratio for liver and kidney of 3.33 and 5.0, respectively, and Pople et al. (1998), ww:dw ratio for liver and kidney of 4.9 and 6.8, respectively. Concentrations for muscle tissue were converted from dw to ww using the ratio from Anan et al. (2001) of 5.0. Due to discrepancies between these ratios for a given tissue type, average tissue concentrations have been reported as a range between the ratios where they have been converted. For direct comparisons, the EC_{50} concentrations from the *in vitro* cytotoxicity assays in this study have been converted to $\mu\text{g/g}$ ww using the density of the media (1.001 g/ml, Supplementary Table S2). The EC_{50} values for the representative skin cell cultures (GT06s-*p* and LT05s-*p*) were used for comparisons to allow for a more sensitive risk assessment and more meaningful interspecies differences to be incorporated. Risk quotients (RQ) were then calculated for contaminants that were cytotoxic ($\text{RQ} = \text{environmental concentration}/\text{EC}_{50} \text{ value}$).

3. Results

The order of sensitivity between the individual cell cultures varied for each compound. However, a skin cell culture was the most sensitive for six out of the ten compounds, and a

heart cell culture was the least sensitive for six out of ten compounds (Table 2). The general order of compound toxicity (from highest to lowest) was $\text{Hg}^{2+} > \text{Cr}^{6+} > \text{Cd}^{2+} > \text{metolachlor} > 4,4'\text{-DDE} > \text{Aroclor 1254} > \text{Cu}^{2+} > \text{PCB 153} > \text{Zn}^{2+} > \text{DEET}$, with some slight differences between cell types. The variability between cell types was generally low for each compound ($\text{RSD} < 40\%$) with the exception of Cd^{2+} ($\text{RSD} = 56\%$) and Cr^{6+} ($\text{RSD} = 70\%$). The variability within each cell type (for the separate assay runs) depended largely on the compound being tested, for example DEET and metolachlor had low relative standard deviations (RSDs) for most cell cultures ($< 20\%$) while Cd^{2+} and Cu^{2+} had high RSDs for most cell cultures ($> 40\%$).

Some of the compounds were not cytotoxic within the limits of solubility in assay media (atrazine 150 μM , phenanthrene 7 μM , diuron 180 μM , dieldrin 0.28 μM , Pb^{2+} 4 mM), and were not included in further tests. Co^{2+} was cytotoxic, however not in a complete ($< 36\%$ decrease in cell viability) or consistent concentration-dependent manner (only concentration-dependent response for skin), thus it was excluded from statistical analyses. Prior to analysis, GT12h-*p* was removed for PCB 153, GT10s-*p*, GT08s-*p* and GT12s-*p* were removed for Zn^{2+} , and GT10o-*p*, GT10l-*p*, GT08s-*p* and GT12s-*p* were removed from Cd^{2+} due to extremely high variation ($\text{RSD} > 70\%$).

Table 2. 24hEC₅₀ values (μM) for the resazurin cytotoxicity bioassay tested on 10 compounds in 13 green turtle cell cultures.

Cells	DEET	PCB 153	Metolachlor	Aroclor 1254	4,4'- DDE	Cu^{2+}	Hg^{2+}	Zn^{2+}	Cd^{2+}	Cr^{6+}
GT10i- <i>p</i>	NR	280	98	NR	130	490	24	1100	59	21
GT10o- <i>p</i>	NR	390	60	NR	110	450	26	990	NR	26
GT10l- <i>p</i>	NR	400	97	NR	170	420	26	870	NR	53

GT10h- <i>p</i>	NR	390	100	NR	190	430	29	1000	120	55
GT06s- <i>p</i>	NR	300	99	NR	100	340	14	830	46	6.3
GT10s- <i>p</i>	NR	490	70	NR	140	280	15	NR	42	18
GT12o- <i>p</i>	6900	310	89	190	68	410	20	560	160	12
GT08s- <i>p</i>	5300	480	97	240	81	250	11	NR	NR	12
GT08i- <i>p</i>	5300	300	110	220	78	300	13	830	65	14
GT08h- <i>p</i>	6000	720	89	260	110	410	20	750	100	38
LT05s- <i>p</i>	5700	320	230	180	70	170	10	390	36	8.2
GT12s- <i>p</i>	5200	380	94	210	70	300	17	NR	NR	60
GT12h- <i>p</i>	7800	NR	100	450	160	310	17	340	54	26
Mean	6047	398	103	251	114	351	18.4	769	76	27
RSD	16%	31%	39%	37%	36%	26%	32%	34%	56%	70%

NR= not reported due to high variation or experimental error

Figure 1. Mean 24hEC₅₀ values for each organ type. Letters denote significance groups after ANOVA (individual turtle nested within organ) and post hoc LSD test. All GT10 cell cultures and GT06s-*p* were excluded from DEET and Aroclor 1254. Some cell cultures were removed from certain compounds due to extremely high variation (see text).

Significant differences between organ types were found for all compounds except Aroclor 1254, Cu^{2+} and Zn^{2+} (Figure 1). When ranked in order of 1 (most sensitive) to 5 (least sensitive), regardless of whether the differences were significant or not, skin was the most sensitive organ type for six out of the ten compounds, one of the two most sensitive organs for nine out of ten compounds, and never had a ranking of four or five. Heart and liver were consistently in the two least sensitive organ types (nine out of ten and six out of seven, respectively). An ANOVA of the ranked order found significant differences between organ types, with skin being the most sensitive and heart and liver the least sensitive ($F=6.431$, $df=4$, $p<0.001$, Figure 2).

Figure 2. Average ranking in order of sensitivity of organ types (1=more sensitive, 5=less sensitive). Letters denote significance groups following ANOVA and LSD post hoc analysis.

Individual turtles contributed significantly to the overall variation for metolachlor ($F=3.366$, $df=7$, $p=0.018$), 4,4'-DDE ($F=6.499$, $df=7$, $p<0.001$), and Cr^{6+} ($F=4.081$, $df=7$, $p=0.004$).

There were no significant differences between the two cells from small intestine (GT08i-p and GT10i-p) for any of the compounds. There were some significant differences between individuals within heart for 4,4'-DDE (GT08h-p differed from GT10h-p and GT12h-p) and

Cr^{6+} (GT12h-*p* differed from GT10h-*p* and GT08h-*p*). GT10 and GT12 within ovary were significantly different for all three compounds (Metolachlor, 4,4'-DDE, Cr^{6+}). Differences between individuals within skin for 4,4'-DDE were driven by differences between GT10s-*p* and the other individuals. Overall, cells from GT10, the turtle with FP, did not stand out as being consistently different from cells originating from healthy turtles.

In vitro species differences (green vs loggerhead turtles) were investigated using GT06s-*p* and LT05s-*p* skin cells and eight of the compounds (DEET and Aroclor 1254 excluded due to inconsistencies). Mean EC_{50} values were significantly higher in green turtles compared to loggerhead turtles for 4,4'-DDE ($F=11.911$, $df=1$, $p=0.026$), Zn^{2+} ($F=26.084$, $df=1$, $p=0.015$), Cd^{2+} ($F=16.548$, $df=1$, $p=0.015$) and significantly lower for metolachlor ($F=30.629$, $df=1$, $p=0.012$) (Figure 3). The lack of difference between species for Cu^{2+} may be the result of high variation for GT06s-*p*; however this level of variation for Cu^{2+} was consistent across all cell cultures with the exception of LT05s-*p*.

Figure 3. Differences between cytotoxicity 24hEC₅₀ values between loggerhead (LT) and green turtle (GT) skin cell cultures for eight compounds. Asterisks denote significant differences.

4. Discussion

Overall, the range of EC₅₀ values was generally small between all the cell cultures (RSD for all compounds <70%), with the biggest difference for Cr⁶⁺ (70%) and the smallest difference for DEET (16%). Comparisons to the literature are limited for many of the compounds tested here, particularly for direct comparisons to sea turtle cell cultures. However, generally the relative toxicity of the compounds tested here support previous finding in the literature for sea turtle and other species cell cultures (Supplementary Table S1). Hg²⁺, Cr⁶⁺, and Cd²⁺ are generally most toxic to human and animal cells, and DEET is the least toxic. DDE, Aroclor 1254, metolachlor, Cu²⁺ and PCB 153 all have similar ranges in toxicity. Zn²⁺ has a large range in toxicity, spanning two orders of magnitude.

4.1 Differences in organ sensitivity

Significant differences were found between organ types for most compounds used in this study. However, the range of mean EC₅₀ values between the organs was not large (RSD <25% for most compounds) and ranged from an RSD of 12% for Cu²⁺ to 67% for Cd²⁺. While the order of sensitivity varied between compounds, an overall pattern of sensitivity was evident. Analysis of the ranked order of the mean EC₅₀s for organ types revealed that skin was consistently the most sensitive organ, while heart and liver were consistently the least sensitive organs. Sea turtle liver has previously been found to be consistently one of the least sensitive organs while heart was one of the least sensitive organs for only Cu²⁺ and Cd²⁺ (Tan et al. 2010, Wang et al. 2013). These studies did not test skin, though found lung or brain (not used in this study) to be consistently one of the most sensitive cell cultures (Tan et al. 2010, Wang et al. 2013). In this study, skin cells performed comparatively to the other organs and could therefore be used in sea turtle toxicology studies as a representative cell culture, particularly for studies on nonspecific (e.g., narcosis, oxidative stress) and reactive endpoints (e.g., protein and DNA damage leading to cytotoxicity). The increased sensitivity of these cells may provide an early detection of effects, which may make them more applicable to conservative environmental risk assessments. Combined with the relative ease of obtaining skin cells from live, healthy animals, turtle skin cell cultures represent a promising avenue to fill in some of the knowledge chasms in sea turtle toxicology.

The nested ANOVAs suggested that the individual turtle from which the cells were established was not a large source of variation between organ types, as individual was only a significant source of variability for three out of ten compounds. Ovary was the only organ that had consistent significant differences between the individuals for all three of these

compounds (metolachlor, 4,4'-DDE and Cr^{6+}). This was overall unimportant when compared to low variation between individuals within other organ types and compounds. Low inter-individual variation has also been found in other cell types and assays, such as human lymphocyte proliferation (Thuvander et al. 1999). Conversely, large inter-individual variation has been found in loggerhead turtle lymphocyte proliferation (Keller et al. 2006) and harbour seal T-lymphocyte proliferation (Neale et al. 2002).

4.2 Healthy vs FP turtle cell cultures

This study found few significant differences between cell cultures derived from a turtle with FP to cell cultures derived from a healthy turtle. While there were no consistent differences between the FP turtle and the non-FP turtles, sample size was low, thus further investigation into potential differences is warranted. It has been suggested previously that cells from FP-diseased turtles may not behave normally. For example, two out of three known aromatase inducers failed to activate in an immortal cell culture derived from the testes of a green sea turtle with FP, suggesting that some pathways may be missing (Keller & McClellan-Green 2004). However, viral DNA decreases quickly in culture and no differences have been found in morphology or growth characteristics between fibroblasts established from tumour cells and normal skin (Work et al. 2009). Thus, cultures derived from FP-diseased turtle may be useful in general toxicity assays. Certainly, tissues from FP-diseased animals are often the easiest to obtain, but their use should be carefully considered.

4.3 Differences in sensitivity between species

GT06s-*p* and LT05s-*p*, cell cultures previously selected to represent green and loggerhead turtles, respectively, were used for *in vitro* species comparisons. Of the four compounds that showed significantly different cytotoxicity between species (metolachlor, 4,4'-DDE, Zn^{2+} and

Cd^{2+}), only metolachlor was more toxic to green turtle skin cells, with the others more toxic to loggerhead skin cells. However, differences in EC_{50} values between species were generally quite similar for all compounds tested here (less than 2-fold difference).

Overall, these results on species comparisons suggest that sensitivity of skin cells from green and loggerhead turtles is generally similar in terms of cytotoxicity. While strong correlations in cytotoxic response *in vitro* can be found between species (Clemmedson & Ekwall 1999), differences between cells from different species are often still evident (Tan et al. 2008, Young et al. 2015, Tong et al. 2016). Cytotoxicity is usually due to non-specific molecular and cellular effects (e.g., changes to membrane fluidity), which are likely to affect most eukaryotic cells in a comparable range of exposure concentrations. Inter-species differences in toxicity are usually visible when other, specific modes of action are involved, for example binding to a species-specific hormone receptor or enzyme, or when species-specific toxicokinetic factors modify the concentration of the contaminant in particular organs. Differences in cytotoxic response between species would thus not reflect differences for other toxicological endpoints (e.g., endocrine disruption, neurotoxicity, etc), for which species-specific differences should also be investigated. Finally, many contaminants have higher accumulation values in loggerhead turtles (e.g. McKenzie et al. 1999, Kaska & Furness 2001, Kampalath et al. 2006, Richardson et al. 2010), further highlighting the need for species-specific assays to fully understand the risk to each species.

4.4 Compounds not cytotoxic to turtle cells

Some compounds tested here did not show cytotoxic effects in sea turtle cells (atrazine, phenanthrene, diuron, dieldrin, Pb). This is supported by the literature for atrazine for rainbow trout adrenocortical cells (Bisson & Hontela 2002), Chinese hamster ovary cells

(Kmetič et al. 2008), human colon cells (Caco-2 cells), white blood cells (WIL2-NS) and human liver cells (C3A) (Leusch et al. 2014). Phenanthrene has been found to be cytotoxic to a rainbow trout gill cell line (RTgill-W1) at concentrations above the solubility limit ($EC_{50} = 177 \mu\text{M}$, Schirmer et al. 1998); however, in our study compounds were only tested within their range of solubility. Diuron also has a low solubility and is thus difficult to test *in vitro* at cytotoxic concentrations (Da Rocha et al. 2013), however has previously been found to have little effect on cell viability in DR-EcoScreen cells (mouse hepatoma Hepa1c1c7 cells with reporter gene) (Takeuchi et al. 2008), human breast cancer cells (MCF-7), choriocarcinoma cells (BeWo) (Huovinen et al. 2015), human colon cells (Caco-2 cells), white blood cells (WIL2-NS) (Leusch et al. 2014). However, diuron was cytotoxic to human liver cells (C3A) at $69 \mu\text{M}$ (Leusch et al. 2014). Dieldrin has been found to be cytotoxic in the fibroblast-like embryonic mouse cell line Balb/c 3T3 ($EC_{50} = 33 \mu\text{M}$, Glden et al. 2002), human cortical neuronal (HCN-2) cells and mouse pancreatic α -endocrine (α -TC) cells ($EC_{50} = 292 \mu\text{M}$, $EC_{50} = 351 \mu\text{M}$ respectively, Kitazawa et al. 2001), however has little effect on cell viability in DR-EcoScreen cells (mouse hepatoma Hepa1c1c7 cells with reporter gene) (Takeuchi et al. 2008). While not found to be cytotoxic in this study, there may be other effects associated with these compounds.

Cobalt was found to be slightly cytotoxic, reducing cell viability by up to 36% for skin (at 20 mM), which was the most sensitive organ. Co^{2+} has previously found to be cytotoxic at lower concentrations than tested here in HaCaT human keratinocytes ($EC_{50} \text{ CoCl}_2 = 475 \mu\text{M}$, Ermolli et al. 2001), human oligodendrocytes (MO3.13) and human gingival fibroblasts (HGF) ($EC_{50} = 215 \mu\text{M}$, $EC_{50} = 706 \mu\text{M}$ respectively, Issa et al. 2008). At present it is unclear if these differences for Co^{2+} are due to actual species and/or organ-specific toxicity, or are an artefact of differences in media and fate within the dosing well.

4.5 Chemical risk assessments

Chemical pollution has been identified as a major threat to sea turtles; however, there remains a paucity of data concerning the effects of pollutants (Wallace et al. 2010, Rees et al. 2016). It is therefore pertinent to place effect concentrations from studies such as this in an environmental context to better identify populations at risk and prioritise research, keeping in mind important limitations of *in vitro* toxicity models (e.g., limited integration of toxicokinetics and higher-order biological events) and in particular cytotoxicity (e.g., focused on non-specific toxicity). Previous sea turtle risk assessments have used effect values from freshwater turtles and birds (Lam et al. 2006, van de Merwe et al. 2009, Dyc et al. 2015); however with increasing amounts of sea turtle *in vitro* data, risk assessments can be more species specific. *In vitro* cytotoxicity studies have shown strong correlations to *in vivo* lethal doses in fish (Schirmer 2006), rodents (Payne et al. 1987, Konsoula & Barile 2005), and humans (Ekwall et al. 1998). *In vitro* bioassays are also increasingly being used in human and ecological risk assessments (Collins et al. 2008, Prasse et al. 2015). However, there are some limitations to these types of comparisons. Concentrations reported in this study and used for comparisons represent nominal concentrations, and are thus overestimations of the bioavailable component (Fischer et al. 2017). Evidence suggests that effect values based on free concentrations rather than nominal concentrations correlate better with *in vivo* effect concentrations (Gülden & Seibert 2005). Cell lines are typically less sensitive than the whole animal (Konsoula & Barile 2005, Schirmer 2006, Tanneberger et al. 2013) suggesting risk assessments based solely on *in vitro* data will likely underestimate effects. Risk quotients presented here are based on accumulation values in particular organs, and therefore represent risk to that organ rather than mortality in the whole animal. Risk quotients were also based on results from acute exposure (24h), which may not be representative of chronic exposure.

Furthermore, EC₅₀ values were used for comparisons, however significant effects take place at much lower concentrations.

Some compounds have not been investigated in turtle blood or tissues and have therefore been compared to exposure values in water, sediment or seagrass. DEET is ubiquitously found in fresh water systems (Costanzo et al. 2007, Aronson et al. 2012), and has been found in seawater up to 1.1 µg/L in Jakarta Bay, Indonesia (Dzikowitzky et al. 2014). These concentrations are well below effect concentrations found here. Similarly diuron, metolachlor and atrazine concentrations are low in the marine environment (Haynes et al. 2000, Lewis et al. 2009, Allan et al. 2017). Aroclor 1254 itself is not often detected in the environment, but it is a mixture of PCBs which are commonly found in sea environments (Müller et al. 1999, Marrucci et al. 2013, Lyons et al. 2015) and animals (Jepson et al. 2016, Stuart-Smith & Jepson 2017). While environmental concentrations of these compounds are generally low, there may be other risks associated with chronic exposure to these compounds, particularly in long-lived species.

The organic compounds, despite having relatively high toxicity, have accumulation values orders of magnitude lower than the EC₅₀ values found in this study (Supplementary Table S3). Risk quotients for the organic compounds were extremely low and are thus not likely exerting negative effects on cell viability at current concentrations, although mixture effects should not be overlooked (Benachour et al. 2007). Some inorganic compounds, namely Cd, Cu, Cr, and Zn, had high RQs in many populations of both species (Table 3). RQs for Hg in green and loggerhead turtles from Japan were extremely high and are cause for concern.

There are some populations that have potentially harmful concentrations of more than one of these compounds and could thus be experiencing mixture toxicity effects, for example those

in Japan (Sakai et al. 1995, Sakai et al. 2000a, Sakai et al. 2000b, Anan et al. 2001), SE Queensland (Pople et al. 1998, van de Merwe et al. 2010a) and the Mediterranean (Kaska & Furness 2001, Franzellitti et al. 2004, Maffucci et al. 2005, Storelli et al. 2005, Andreani et al. 2008, García-Fernández et al. 2009). Many of these compounds, including those that were not cytotoxic or are only cytotoxic well above exposure and accumulation values, may elicit other negative effects at lower concentrations, such as endocrine disruption (Henson & Chedrese 2004, Russart & Rhen 2016), neurotoxicity (Grandjean & Landrigan 2006, Catalani et al. 2011), immunotoxicity (Keller et al. 2006, Desforjes et al. 2016), oxidative stress (Valko et al. 2005, Labrada-Martagón et al. 2011) and genotoxicity (Marabini et al. 2011, Sharma & Vig 2012). Thus, these compounds should further be tested against a wide range of endpoints to better assess risks posed to turtle populations.

Table 3. Range of risk quotients for cell viability above 1 in different tissue types based on accumulation values in green and loggerhead sea turtles. Locations with the highest RQ have been included in the last column.

Compound	Species	Tissue type	Range of RQ	Location(s) with highest RQs
Copper	Green	Liver	1.73 – 4.2	SE Queensland, Australia
	Loggerhead	Liver	1.4 – 1.7	Cape Ashizuri, Kochi Prefecture, Japan
Cadmium	Green	Kidney	1.1 – 9.4	Gladstone, Australia
	Loggerhead	Kidney	1.1 – 9.6	Cape Ashizuri, Kochi Prefecture, Japan
Chromium	Green	Muscle	1.3 - 16	W Gulf of Mexico, US
		Kidney	1.0	Hawaiian Islands, US; Gladstone, Queensland
	Loggerhead	Liver	1.7 - 34	NW Indian Ocean
		Liver	1.5 – 2.0	Mediterranean coast, Turkey
Zinc	Green	Liver	1.1	Yaeyama Islands, Japan
	Loggerhead	Liver	1.0 – 1.1	SW Mediterranean coast, Spain; Cape Ashizuri, Kochi Prefecture, Japan
		Kidney	1.2	Portugal
		Muscle	1.3	Mediterranean and Adriatic Sea

Mercury	Green	Ovary	1.5	Cape Ashizuri, Kochi Prefecture, Japan
		Fat	2.6 – 2.7	North Adriatic Sea
	Loggerhead	Liver	26.4 – 103.8	Haha-Jima Island, Japan
		Liver	200 - 4075	Cape Ashizuri, Kochi Prefecture, Japan

Abbreviations: RBC: red blood cells

For species such as sea turtles, where obtaining *in vivo* effect values is an impossibility, *in vitro* data provides a useful avenue for identifying and prioritising research into chemical risk to these species. As the field of sea turtle toxicology continues to grow, results from studies such as this provide crucial information for the careful design of future experiments and guiding management and conservation plans.

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6. Declaration of interest

None.

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Graphical Abstract

Highlights:

- There is a pattern of organ sensitivity in cell cultures
- Cell cultures from skin were the most sensitive, liver and heart were the least
- Primary skin fibroblasts may be a suitable representative cell type
- A risk assessment identified several populations at risk from Zn, Cd, Cr, Hg and Cu

ACCEPTED MANUSCRIPT