Assessing the impact of benzo[*a*]pyrene with the *in vitro* fish gut model: an integrated approach for eco-genotoxicological studies

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List of abbreviations:

PAH	Polycyclic aromatic hydrocarbon
B[*a*]P	Benzo[*a*]pyrene
APH	Acid phosphatase
LDH	Lactate dehydrogenase
EROD	7-Ethoxyresoorufin-O-deethylase
GST	Glutathione Transferase
AOP	Adverse Outcome Pathway
ADME	Adsorption, Distribution, Metabolism and Excretion
TEER	Trans-epithelial Electrical Resistance
DNA	Deoxyribonucleic Acid
3Rs	Replace, Reduce, Refine
ECVAM	European centre for the Validation of Alternative Methods
GC-MS	Gas Chromatography-Mass Spectrometry
L-15	Leibovitz medium
FBS	Foetal bovine serum
TRIS/HCL	Tris hydrochloride
NADH	Nicotinamid adenine dinucleotide
GST	Glutathione transferase
β-NF	β-naphthoflavone
α-NF	α-naphthoflavone
DMSO	Dimethyl sulfoxide
PCB	Polychlorinated biphenyl
OECD	Organisation for Economic Co-operation and Development
AHR	Aryl hydrocarbon receptor

Abstract

In vitro models are emerging tools for reducing reliance on traditional toxicity tests, especially in areas where information is sparse. For studies of fish, this is especially important for extrahepatic organs, such as the intestine, which, until recently, have been largely overlooked in favour of the liver or gill. Considering the importance of dietary uptake of contaminants, the rainbow trout (*Oncorhynchus mykiss*) intestine-derived cell line RTgutGC was cultured, to test its suitability as a high-throughput in vitro model. Benzo[*a*]pyrene (B[*a*]P) is an important contaminant and a model polycyclic aromatic hydrocarbon (PAH). Over 48 h exposure, a range of endpoints and xenobiotic metabolism rates were examined at three different pH levels indicative of the in vitro (pH 7.5) and in vivo (pH 7.7) and hind-gut (pH 7.4) regions as a function of time. These endpoints included (i) cell viability: acid phosphatase (APH) and lactate dehydrogenase (LDH) assays; (ii) glucose uptake; (iii) cytochrome P450 enzyme activity: 7-ethoxyresoorufin-O-deethylase (EROD) assay; (iv) glutathione transferase (GST) activity; (v) genotoxic damage determined using the comet assay. Absence of cell viability loss, in parallel with decrease in the parent compound (B[*a*]P) in the medium and its subsequent increase in the cells suggested active sequestration, biotransformation, and removal of this representative PAH. With respect to genotoxic response, significant differences were observed at both the sampling times and the two highest concentrations of B[*a*]P. No significant differences were observed for the different pH conditions. Overall, this in vitro xenobiotic metabolism system appears to be a robust model, providing a basis for further development to evaluate metabolic and toxicological potential of contaminants without use of animals.

Keywords: 3Rs; rainbow trout; RTgutGC; dietary exposure; B[*a*]P; comet assay

1 Introduction

Aquatic toxicity and bioaccumulation studies are important components of environmental hazard and risk assessment of chemicals. The behaviour of a compound in the environment is primarily assessed using bioaccumulation studies in aquatic organisms, bioaccumulation being the result of absorption, distribution, metabolism, and excretion (ADME) processes [1]. For most regulatory assessments, broad suites of in vivo toxicity tests continue to provide basic information for the decision-making process. The time and resources necessary to support this approach run counter to the demands being faced at present. Furthermore, there is mounting pressure to minimise animal usage and to use animal-free approaches in initial testing strategies, where possible [2– 5]. For many years, assays using cell lines derived from fish have been proposed as alternatives to animal use in aquatic toxicity testing with excellent reviews on the topic available [6–11]. More recently, they have been proposed as in vitro-in vivo toxicity extrapolation tools [12–15], in addition to adding information complimentary to adverse outcome pathway (AOP) predictions [16].

In vitro systems are most useful if they have predictive power for in vivo outcomes and, as such, should be developed based on physiological comparability to the organ of choice. In essence, the cell culture system should mimic a particular tissue and represent a simplified, miniaturised copy of the organ or tissue of interest. These systems offer the possibility of addressing and answering fundamental questions; facilitating high-throughput toxicity screening; and providing reproducible results, due to a standardised and well-characterised (although artificial) environment. The results of such experiments can subsequently be extrapolated to complex natural systems, thereby providing a tremendous reduction in reliance on traditional animal tests. The models allow studies at the cellular and subcellular levels to be carefully probed. One of the driving forces of their widespread adoption is their potential ability to aid in the reduction and replacement of animal experiments during toxicity assessments. As such, they have become popular for characterising initial mechanisms of action of toxicity, since the primary interaction between chemicals and biota occurs at the cellular level. Early manifestations of toxicity can be used to evaluate pollutant exposure [7] and have previously been reported as a sensitive and reliable assessment tool in aquatic systems [10, 17]. These studies have primarily focused on the use of typical xenobiotic-metabolising organs, such as the liver and gill in rainbow trout (*Oncorhynchus mykiss*), a model fish species [11, 13, 14, 18–22], but are readily available from numerous fish species [23].

There is growing concern over the presence of genotoxic, carcinogenic, and reproductive contaminants in the aquatic environment [24]. Genotoxic compounds could potentially affect the short- and long-term survival of the species while also posing risks to human health via the food chain [25, 26]. Genotoxicity could be linked to various phylogenetic endpoints [26–29]. It is therefore important to characterise thoroughly the metabolic capabilities and properties of a model system before it is widely accepted to assess the toxic potential of

environmental contaminants. Despite the identification and localisation of P450-dependent enzymes in the fish intestine and similarly in the liver, indicating their dual metabolic activity [30, 31], in vitro models of the intestinal system are relatively few. In aquatic systems, one cell line originating from the intestine of rainbow trout is readily available (RTgutGC; [32]). In early work, major histocompatibility genes were identified in this cell line [33]. Later work demonstrated trans-epithelial electrical resistance (TEER) comparable to values measured in vivo; apical localisation of tight junction proteins; and the induction of Na^+/K^+-ATPase mRNA following exposure to saltwater buffer [34]. Recent work published by our laboratory has demonstrated the direct comparability of this model to existing ex situ gut sac preparation methods when exposed to copper, a ubiquitous environmental contaminant [35]. However, the suitability of this cell line for toxicological assessment and as an in vitro animal replacement tool needs to be further examined using more general environmental contaminants, especially organic contaminants which require metabolic activation, to establish its sensitivity and comparability before incorporation into a regulatory framework.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the aquatic environment, with their metabolism well studied in a variety of fish and mammals. This is due in large part to the observation that the compound benzo[*a*]pyrene (B[*a*]P) requires metabolic activation to bind to DNA and other biomolecules to induce biological response. The mutagenic and carcinogenic properties of this compound are initiated by cytochrome P450-dependent monooxygenase activity [30, 36] and, due to its lipophilic nature, it is likely that fish are exposed to large doses via the diet [37–40]. Indeed, a recent study reports that the intestine is the first barrier for PAH uptake from the diet [41].

Development of an in vitro cell-based model from the intestine of fish that mimics its mechanical, structural, absorptive, transport, and other pathophysiological properties has the potential to accelerate toxicity screening by providing a high-throughput platform to assess the ecotoxicological potential of a compound. This concurrently aims to reduce the number of animals used in traditional toxicity tests, in line with “3Rs” (replacement, reduction and refinement) principles. The primary objective of this study was to test whether the RTgutGC cell line can be used as robust environmental monitoring tool. As the bottleneck in in vitro toxicological studies is reported by ECVAM to be functional metabolism [42], we focussed on characterising metabolism and evaluating genotoxic damage within this system, building up from our previous study, which included thorough morphological and histological characterisation of this cell line [35]. In this work, uptake of a known genotoxicant and carcinogen, B[*a*]P), was verified using Gas Chromatography-Mass Spectrometry (GC-MS) quantification of the removal of the parent compound and immunofluorescence identification in the cells. Furthermore, external pH is known to partly determine the cytoplasmic or intracellular pH and can have a subsequent effect (beneficial or detrimental) on enzymatic activity, reaction rate, protein stability, and other biological cascade processes, across numerous taxonomic groups. Following initial characterisation of the

activity, therefore, B[*a*]P exposures were also carried out using three exposure solutions with varying pH (L-15 medium pH 7.5 and a L-15: saline exposure of pH 7.7 and 7.4) as detailed in our earlier studies [35]. Using B[a]P as model environmental contaminant, we probed the ability of this cell line for active xenobiotic metabolism, the resulting genotoxicity, and its comparability to information available in the literature.

2 Materials and methods

2.1 Experimental design

Prior studies, including our own, have established the comparability of this intestinal in vitro model to native tissue cultured as monolayers on Transwell inserts [34, 35]. In this study, we focused on the metabolic activity of the system following exposure to a known carcinogen, B[*a*]P, as mentioned above. Experiments were carried out in two stages, with stage I testing whether the cell line carries out active uptake of B[*a*]P, using epifluorescence microscopy and an analytical technique. Following confirmation of uptake of this compound, basic characterisation of the response of the cell line to this agent was carried out (i.e., cell viability assessed with the acid phosphatase assay (APH) and lactate dehydrogenase (LDH) release). In stage II, parallel studies determined glucose depletion, CYP1A activity (EROD assay) and GST activity. In addition, genotoxic response was determined using the alkaline single cell electrophoresis or comet assay. As this study was carried out to ascertain the potential of this method to act as a screening tool for environmental contaminants, these experiments were not carried out on Transwell inserts as done in our earlier studies [35] and are instead representative of cells grown as monolayers on 12-well plates.

2.2 *Chemicals and medium*

All reagents and chemicals used in the experiments were obtained from ThermoFisher (UK) or Sigma-Aldrich (UK) unless otherwise indicated. B[*a*]P was obtained from Sigma (B1760; CAS:50-32-8). Stock solutions of B[*a*]P (500 μM in 100% cell culture grade DMSO) were prepared prior to the initiation of experimentation, aliquoted, and stored at -20°C. For each experimental repeat or new assay, a new aliquot was used. Secondary saline exposure solutions used a modified Cortland saline solution previously described [28]; two pH values of a saline solution were chosen to represent the mid (pH 7.7) and posterior intestine (pH 7.4). Unconditioned unmodified L-15 medium was also run in parallel (pH 7.5). As pH can vary over time, the cell culture experiment was set up as per the exposure scenario. Briefly, exposure stocks made in L-15 medium, L-15 medium: saline (pH 7.7 and 7.4) were added to each well of a 12-well plate and monitored over 48 h. Sampling was done at 24 and 48 h, with exposure samples checked both using pH test strips (Sigma-Aldrich) and with a pH meter (FisherBrand Hydrus B500) at incubation temperature (21 °C). There were no significant differences between the pH values (pH 7.7 +/- 0.06 and 7.4 +/-0.08) of exposure solutions ($p = 0.65$) without cells and so the experiment was also repeated

with RTgutGC cells. Data are presented as the pooled means of all exposure solutions with the average breakdown of pH 7.7 and 7.4 as follows: at 24 h, 7.5 ± 0.13 and 7.4 ± 0.04 and at 48 h, 7.5 ± 0.11 and 7.4 ± 0.02, respectively. There were no significant differences between pH values when compared to initial exposure medium: saline stocks ($p = 0.88$). As such, experiments were not additionally buffered during the exposure period.

2.3 *Cell line maintenance*

The rainbow trout gastrointestinal cell line RTgutGC [32] was a kind gift from Dr. Lucy Lee (University of the Fraser Valley, Abbotsford, BC, Canada). The cell line was routinely cultured in 75 cm^2 culture flasks at room temperature (21 °C) in L-15 culture medium supplemented with 10% foetal bovine serum (FBS). Cells were seeded normally at a density of 5×10^4 cells/mL, and became confluent after 7-9 days. Trypan blue (1:1 ratio to cells) was used to assess cell viability prior to experimental set up and samples with viability <95 % were discarded. Unless otherwise indicated, experiments were carried out on 12-well plates at a seeding density of 10×10^4 cells/mL and cells were allowed to attach and become confluent over a three-day period. A half medium exchange (L-15 without FBS) occurred on the day prior to exposure (FBS reduced to <5 %). On the day of exposure, half of the medium was exchanged for the exposure stocks of B[*a*]P (1:1 split). Through consecutive medium exchanges, the final FBS concentration in the medium used throughout the biochemical assay analysis was <2.5 % (not accounting for cellular usage).

2.4*B[a]P concentration in medium and cell samples; determination by GC-MS*

Two preliminary investigations were carried out to determine whether the cell line could take up this prototypical PAH. Firstly, RTgutGC uptake of B[*a*]P was confirmed using an epifluorescence microscope, where cells were seeded onto Nunc Lab-Tex^R II chamber slide system (C7057; Sigma-Aldrich, UK), allowed to become confluent, and washed twice with DPBS prior to exposure. Following exposure, cells were incubated in varying concentrations of B[*a*]P (0-50 μg/L) for 24 and 48 h in the dark, fixed with 4% formal saline, and cells stained with DAPI for 30 s (1 μg/L; Sigma-Aldrich, UK). Images were obtained using a Nikon epifluorescence microscope (Eclipse 80i) with camera attachment (DS-Qi1Mc). Images were captured and processed using the NIS elements application suite (Nikon) where brightness and contrast were adjusted. Upon examination, the epifluorescence microscopy showed that B[*a*]P was notably detected in the RTgutGC cells at all concentrations, without exception.

Following microscopic verification of uptake of B[*a*]P, cells were seeded at the aforementioned densities and allowed to become confluent in T-175 cm^2 tissue culture flasks (660160; Greiner Bio One, UK). Controls, i.e., flasks without cells but with B[*a*]P-spiked unconditioned L-15 medium were processed alongside each experimental repeat to quantify adsorption to plastic ware ($n = 4$). The B[*a*]P analysis was based on a protocol previously developed and validated in our laboratory [43]. The aqueous medium samples (10 mL) were pipetted

from the cell culture bottle into 15 mL analytical/chromatography glass vials (Sigma-Aldrich, UK) and dichloromethane (2 mL, 650463, Sigma-Aldrich, UK) added. Cell samples were scraped into pre-weighed glass vials to standardise reporting of data to the literature. B[a]P-d_{12} (100 ng in 20 μL acetone) was then added as an internal standard. The medium was collected as above, due to the fact that DCM was shown to dissolve the polystyrene cell culture flasks during preliminary characterisation. Following thorough shaking, the mixtures were stored in the dark at 4C. Immediately prior to analyses, the DCM layers were removed into glass micro-vials. Residual water was removed by adding pre-cleaned anhydrous sodium sulphate to the extract. Cell samples were transferred by scraping into pre-weighed vials and spiked with the internal standard B[a]P-d_{12} (100 ng in 20 μL acetone). Dichloromethane (2 mL) was added and the cells were extracted in a sonication bath for 20 min. The supernatants were finally dried with pre-cleaned anhydrous sodium sulphate. Aliquots (1 μL) of the sample extracts were analysed using an Agilent Technologies 7890A GC system interfaced with an Agilent 5975 series Mass Selective detector (Agilent Technologies, United Kingdom). A Restek Rxi-1MS (crosslinked poly dimethyl siloxane) capillary column (30 m) with a film thickness of 0.25 μm and internal diameter 0.25 mm was used for separation, with helium as a carrier gas (maintained at a constant flow rate of 1 mL/min). Extracts were injected splitless, with the injector maintained at 250 °C. The oven temperature programme was 40 °C for 2 min and then increased at 15 °C/min to a final temperature of 250 °C, where it was held for 4 min. The mass spectrometer was operated in electron impact mode (at 70 eV) with the ion source and quadruple analyser temperatures fixed at 230 °C and 150 °C, respectively. Samples were screened for B[a]P and B[a]P-d_{12} using selected ion monitoring, with target ions 252 (253 and 126 for confirmatory purpose) and 264, respectively. Prior to sample extract analyses, the system was calibrated using authentic standards. With each batch of samples, a solvent blank, a standard mixture, and a procedural blank were run in sequence for quality assurance purposes. In addition, B[a]P adsorption to plastic ware during cell sample exposure was quantified and accounted for during analysis. B[a]P concentrations were calculated based on the internal standard.

2.5 *Cellular assays to evaluate cytotoxic action*

2.5.1 *Lactate dehydrogenase (LDH) activity*

The method for release of LDH into the culture medium was adapted from early methods used in our laboratory [35, 44], which were modifications of an earlier protocol [45]. Following experimental setup in a 12-well plate as previously outlined, a 50 μL aliquot of medium (following B[a]P exposure) was added to each well of a 96 well-micro plate in triplicate on ice, followed by 250 μl of reaction buffer (50 mM TRIS/HCL and 0.14 mM NADH; pH 7.5). The plate was incubated at room temperature (22 °C) for 5 min and the reaction started with the addition of 25 μl of 12.1 mM sodium pyruvate dissolved in 50 mM TRIS/HCl buffer (pH 7.5). Plates were mixed thoroughly, and enzyme activity recorded for 25 min at 25 °C in a micro-plate reader (FLUOstar Omega, BMG Labtech, UK) at

340 nm. LDH was calculated using the Beer-Lambert law and LDH enzyme concentration expressed as LDH release (nmol min^{-1} mL^{-1} mg $protein^{-1}$).

2.5.2 *Determination of cell viability*

Cell viability was assessed as per methodology previously established for mammalian cell lines, based on the quantification of cytosolic acid phosphatase activity (APH assay) [46]. Briefly, monolayer cultures (10,000 cells per well) were treated with B[*a*]P for 48 h over a range of concentrations. Following treatment, cells were washed thrice with DPBS and re-suspended in 100 μL DPBS prior to the addition of 100 μL fresh prepared reaction buffer protected from light (2 mg/mL *p*-nitrophenyl phosphate (Fisher) in 0.1 % v/v Triton-X-100 in 0.1 M sodium acetate buffer, pH 4.8). Following incubation for 2 h at room temperature (21°C) under gentle rotating (50 rpm), 1 M sodium hydroxide (10 μL), was added to each well and absorbance was recorded at 405 nm using an automated 96-well plate reader (FLUOstar Omega, BMG Labtech UK). Samples were run in triplicate, in addition to a blank buffer control which was subtracted prior to calculations. Viability was expressed as percentage of control (unexposed cells in L-15 medium) after correction for fluorescence from the incubation buffer.

2.5.3 *Determination of glucose activity*

Glucose concentrations in the intestinal cell line were determined using an enzyme glucose kit (GAG020; Sigma) as per the manufactures instructions. The method was modified to allow for use with a micro-plate reader in a 96-well assay format as previously reported for spheroids in our laboratory [44]. Briefly, an aliquot (40 μL) of medium was added to each well in triplicate of a 96-well plate and assay reagent (Glucose Oxidase/Peroxidase reagent + *o*-dianisidine reagent), 80 μl, was added and mixed. A glucose standard curve was run on each plate to account for interplate variability. The plate was incubated for 37 °C for 30 min, protected from light. The reaction was stopped by the addition of 12 N H_2SO_4, 80 μL, and the absorbance measured on a micro-plate reader (FLUOstar Omega, BMG Labtech UK) at 540 nm. Results were expressed as μmol^{-1} mL^{-1} min, and the rate of glucose uptake calculated (from 0 - 24 h).

2.6 *Determination of 7-ethoxyresorufin-O-deethylase (EROD) activity*

EROD activity, used as a marker of CYP1A function, was performed using a well-established methodology [21], with modifications specifically developed in our laboratory. In preliminary experiments, a positive-control CYP1A inducer (β-naphthoflavone (β-NF), CAS 6051-87-2; 50 μL; final well concentration, 0.36 μM), CYP1A inhibitor (100 μM α-naphthoflavone; CAS 604-59-1) and a solvent control (0.1% DMSO; final well concentration) were added to each well (dissolved in serum free L-15 medium) and incubated at 21 °C for 48 h. EROD activity was assessed as below prior to B[*a*]P exposure, to ensure CYP1A activity in this cell culture model.

After establishment of CYP1A activity, RTgutGC cells were exposed to B[*a*]P for 24 - 48 h in a black 96-well plate in order to determine the ability of this compound to induce EROD activity in this intestinal culture system (seeding density, 10×10^4 cells/well). Exposure was initiated by removing the culture medium (150 μL) from confluent RTgutGC cells (leaving 50 μL in 96 well plate) and replacing it with 50 μL exposure medium (L-15 or L-15:saline) to which varying B[*a*]P concentrations had already been added.

Following incubation, 50 μL reaction buffer (serum free L-15 containing 16 μM 7-ethoxyresorufin (7-ER), 18 μM dicoumarol; final well concentrations; pH 7.4) was added to each well (final volume, 150 μL) and the production of resorufin measured using a fluorescence plate reader (FLUOstar Omega, BMG Labtech UK; excitation = 544 nm; emission = 590 nm) immediately following the addition of the medium and every minute thereafter for 60 min. Standard curves of resorufin (0 - 256 pmol well^{-1}) were run for each individual plate (150 μL volume). Total protein content of the cell extract was determined with the fluorescamine assay [47] with modifications [48] with bovine serum albumin (BSA) used as standard (1 - 0.0078 mg/mL). Following fluorescence recording, each well was rinsed thrice with PBS, and re-suspended in 100 μL PBS and frozen for 45 min at -80 ˚C. After defrosting at room temperature, fluorescamine solution (0.3 mg/mL in acetone, 50 μL) was added to each well and incubated for 10 min in the dark. Fluorescence was recorded at 390 nm excitation and 460 nm emission, respectively. EROD activity was subsequently expressed as picomoles of resorufin per min per miligram of protein (pmol/min/mg protein) with each sample run at a minimum in triplicate on each plate.

2.7 *Determination of glutathione transferase (GST) activity*

Total GST activity following B[*a*]P exposure was assayed in the RTgutGC cellular lysate with CDNB as substrate as described in the manufacturer's instructions (CS0410; Sigma). Results were expressed as μmol^{-1} mL^{-1} min.

2.8 *Determination of genotoxicity*

Genotoxic response of the RTgutGC cells following B[*a*]P was assessed using single gel electrophoresis and performed using protocols previously established in our laboratory using various fish cell lines [27–29]. B[*a*]P requires metabolic activation by cytochrome P450 enzymes to induce genotoxicity and as such this assay could be considered as a surrogate for the indirect measurement of metabolic activation in this cell line. As such, cells were cultured as normal until 100% confluent and exposed to a range of B[*a*]P concentrations. In each experiment, cell viability was assessed using the Trypan blue dye exclusion assay. Only samples with viability >90% were used.

In brief, prior to the experimental exposure, slides were pre-coated with normal melting point agarose (NMPA; 1.5% in 1X TAE buffer) and allowed to set. Cells were removed from the wells using trypsin and a subset re-suspended in 1.5% low-melting-point agarose (LMPA; 0.75% in PBS), coversliped, and dried at 4 ˚C. Slides were immersed in lysis solution (2.5 M NaCL, 100 mM EDTA, 10 mM Tris, 1 % N-lauryl-sarcosine, 1 % Triton X-

100, 10% DMSO, pH adjusted to 10 with NaOH) for 1 h at 4 °C and then placed in pre-chilled electrophoresis buffer to unwind (1 mM EDTA, 0.3 M NaOH, pH 13). Electrophoresis was performed at 25 V, 620 mA for 25 min (1.25 V/cm) (Compac-50 HTP Comet Assay Tank, Cleaver Scientific, UK). Following electrophoresis, slides were transferred to neutralisation buffer (0.4 M Tris, adjusted to pH 7 with HCl) for min, rinsed (×3) with distilled water, and allowed to air dry. Slides were scored using an epifluorescence microscope (DMR; Leica Microsystems, Milton Keynes, UK) and imaging system (Comet IV, Perceptive Imaging, UK) where 50 cells per microgel (100 cells per slide) were analysed per treatment. Slides were coded and randomised to ensure unbiased scoring. Comet assay software packages record a number of different parameters, with % tail DNA considered the most reliable [49]. Hence, comet assay results are reported as mean % tail DNA.

2.9 *Statistical analysis*

In all experiments, "n" refers to the number of non-parallel passages of the cell line. Statistical analysis was preformed using R, Version 3.1.3 [50]. Data was tested for normality using the Shaprio-Wilk test (SW) or Anderson Darling normality test (AD), with homogeneity of variance evaluated using Levene's test (L) and manual examination of QQ-plots. Where assumptions were met, data was analysed using a t-test or two way ANOVA with Tukey's pairwise comparisons as Post-hoc. When assumptions of normality were not met, the non-parametric Kruskal Wallis test was used followed by Dunn's pairwise comparison with Bonferroni correction for multiple comparisons. Data was presented as mean ± standard deviation unless otherwise indicated. Box and whisper plots were used to display the data giving a summary of the variables in question in the form of median values, quartiles, range and possible extreme values (outliers). Significance was set at $p < 0.05$ (*), although in some instances for highlight significant results, $p < 0.001$ (***) is also reported.

3 Results

3.1 *B[a]P concentration in medium and cell samples, determined by GC-MS*

Three experimental exposures of plastic-ware (without cells) and the RTgutGC cell line exposed to 0.2 μM (~5.4 μg/L) B[*a*]P were carried out, where concentration was measured in cells and medium at 24 and 48 h to elucidate variation in adsorption and absorption of B[*a*]P to plastic-ware and individual intestinal cells. Each experiment was performed in duplicate, with results presented as the mean ± standard deviation ($n = 3$). A representative chromatograph of cell dichloromethane extract is presented in Fig. 1a with the signal of the B[a]P ion m/z 252 (single ion monitoring) in the cell extract presented in Fig. 1b. B[*a*]P uptake in the cell culture model was calculated with respect to a concurrent experiment elucidating adsorption of B[*a*]P to the cell culture plastic-ware (~ 12%) and uptake rates in cells corrected for this fraction. An average measurement of 0.0057 ± 0.0020 B[*a*]P μg/g in cells was recorded at 24 h, with 0.0071 ± 0.0036 B[*a*]P μg/g recorded at 48 h. An independent samples t-test was conducted to compare differences in B[*a*]P in cells and medium over time, with

criteria for normality met by the samples (p = 0.71). The degree of B*a*]P uptake was markedly different in the cells (n = 3, CV = 34 %, 18 % difference between 24 and 48 h) but was not statistically significant (t = -0.45, p = 0.69). When B[*a*]P depletion was examined in the medium (n = 3, CV = 59 %, 58 % difference between 24 and 48 h), there was a marked difference in B[*a*]P present in the medium, but again this is not significant (t = 1.34, p = 0.31) (Fig. 1c).

3.2 *Cellular assays to evaluate cytotoxic action*

3.2.1 *LDH release*

Three experiments were run to investigate damage to the cellular membrane via the LDH assay following B[*a*]P treatment (0.02 - 100 μM) over a 48 h exposure period. Data was analysed using a two way ANOVA (with pH, time and concentration as factors) due to assumptions being met (AD: p = 0.14; L: p = 0.08). No significant difference was identified between concentrations (p = 0.52), pH (p = 0.13) or time (p = 0.59).

3.2.2 *Cell viability*

Deleterious effects on cell viability measured using the APH assay was investigated in three non-parallel experiments following B[*a*]P exposure (0.02 - 100 μM) over a 48 h period. Due to non-normality, data was analysed using the Kruskal Wallis test with Bonferroni corrections and revealed no significant differences between concentrations, pH or exposure time.

3.2.3 *Glucose activity*

Following a lack of significant differences between sampled time points for cell LDH activity and cell viability, questions arose regarding sampling time points. We hypothesized that later sampling times may obscure earlier responses by the cell, and that minimal activity at 24 h (in terms of the biochemical assays) reflects cellular activity at plateau/ saturation. Transepithelial transport is an energy-consuming process; hence, trends in glucose levels may be used as an indicator of cells' increased energy requirements, or may indicate when B[*a*]P absorption/uptake is likely to occur. For this purpose, three experiments were evaluated for variation in glucose measured in the medium following exposure to a range of B[*a*]P concentrations (0, 0.02, 0.2, and 2 μM) over a period of 24 h. Samples were taken at 0, 1, 2, 4, and 24 h. A Shapiro-Wilk test revealed that the distribution of glucose within each group was normally distributed following a log transformation, while a Levene's test revealed homogeneous variances. Following pre-processing of data, a two way analysis of variance (ANOVA) on the outlined factors yielded significant variation among the conditions of pH (F = 3.63, $p < 0.05$) and time (F = 3.84, $p < 0.001$). A post hoc Tukey test on pH revealed significance was driven by parallel pH alone (7.5 and 7.4)($p < 0.05$), while significance over time was driven by differences in mean over time when compared to the 24 h time point ($p < 0.05$). Although there were no significant differences between B[*a*]P concentrations (p =

0.12), a trend in glucose depletion in medium samples (Fig. 2) was seen where parallel pH values concurrently see the highest glucose depletion at 2 μM B[a]P (Table I). This is opposite to the higher pH of 7.7 which sees this occur at a B[a]P concentration of 0.2 μM.

3.3 *EROD activity*

Initially, preliminary experiments were carried out to confirm the presence of CYP1A in the RTgutGC cell line using the EROD assay. RTgutGC cells grown in wells (black) 96 well plates (5 d) were first exposed to the control CYP1A inducer β-naphthoflavone at a range of 0.36 - 2.6 μM in addition to simultaneous co-exposure with the CYP1A inhibitor α-naphthoflavone. Data was log transformed as it did not meet assumptions of normality and homogeneous distributions. The significant induction and inhibition of CYP1A activity in the presence of βNF concentrations (induction) and co-exposures of βNF with α-NF (inhibitor; 100 μM) are shown in Table 2. Inhibition ranges from 54 % in singularly exposed samples (α-NF) to up to 94% in co-exposes relative to solvent control (without α-NF). The active inhibition of CYP1A supports the presence of this cytochrome in the cell line and as a consequence was subsequently exposed to B[a]P.

Following the exposure of B[a]P to cells, EROD induction was determined at 24 and 48 h. As with the preliminary experiment, data was log transformed due to non-normality. A typical dose response curve was observed (Fig 3) with a maximal induction of EROD activity in L-15 medium recorded at a concentration of 0.2 μM B[a]P following 48 h exposure. However, this trend is more variable under saline conditions, where maximal EROD response was recorded at 10 μM following 48 h exposure under pH 7.7 and pH 7.4, respectively. This maximal induction corresponds to an inhibition of EROD activity of 80% (0.395 ± 0.12 pmol/min/mg protein) and 83% (0.312 ± 0.14 pmol/min/mg protein) for pH 7.7 and 7.4 when compared to L-15 medium alone (1.95 ± 0.12 pmol/min/mg protein), a trend which is visible within all concentrations. Although significant differences were observed between pH's (ANOVA; $n = 3$, $p < 0.001$), concentration (ANOVA; $n = 3$, $p < 0.05$) and incubation time (ANOVA; $n = 3$, $p < 0.001$), Tukey's post hoc test revealed significance was limited to differences between medium and saline exposures ($p < 0.001$).

3.4 *GST activity*

Three experiments were carried out to analyse GST activity following 24 h and 48 h exposure to a range of B[a]P concentrations in L-15 medium and a 1:1 ratio of L-15 medium to Cortland saline (pH 7.7 and pH 7.4 respectively). GST data were normally distributed (AD; $p = 0.15$) with homogenous variances (L; $p = 0.39$). Application of a two way ANOVA revealed pH as the only significant factor with significant ($p < 0.05$) differences identified in parallel pHs of 7.5 (L-15 medium) and pH 7.4, respectively. An example of GST activity in the RTgutGC cell line following B[a]P is presented in Fig. 4.

3.5 *Genotoxicity*

Five experiments were conducted to elucidate the genotoxic response to B[*a*]P, using our multi-factor experimental design. Due to non-normal data and non-homogenous variances, data was arcsin transformed. Significant differences were identified between the two sampling time points (ANOVA, $p < 0.001$) and between some B[*a*]P concentrations but not all. Significant differences (with respect to solvent controls/exposure solutions) were only observed in the upper concentrations of 10 and 50 μM (Fig. 5) at both 24 and 48 h. No significant differences were observed between the three pH values/solvents investigated ($p = 0.58$).

4 Discussion

In the aquatic environment, diet is one of the most important routes of exposure to the common contaminant B[*a*]P. Previous studies have demonstrated its presence in the diet and tissue of numerous organisms in addition to its biotransformation by lower organisms and subsequent food chain transfer [26, 39]. Studies have also confirmed active uptake and metabolism of this compound via the diet of fish [51, 52], vertebrates [53] and mammals [54]. To our knowledge, the current study represents the first report of xenobiotic metabolism, using this ubiquitous contaminant (i.e. B[*a*]P), in the intestinal RTgutGC cell line. For metabolic comparability of any in vitro model system to native tissue, the inducing compounds must first be shown to cross the membrane barrier which may lead to alterations in the intracellular availability of the inducer. Indeed, should detoxification occur via a particular pathway, this will lead to a subsequent decrease in the availability of inducer necessary to elicit a measurable response. The uptake of B[*a*]P was shown in the RTgutGC cell line through chemical analysis of substrate depletion. In addition, metabolic induction via the cytochrome P450 pathway (EROD activity) was also confirmed. The increase of B[*a*]P in cells after 48 h exposure in combination with decreased B[*a*]P measured in the medium suggest active enterocytic transport and transformation of B[*a*]P in this model, although it was not feasible during the study to identify the metabolites produced. We hypothesise that the cells eject B[*a*]P back into the medium in a non-toxic form as a means to prevent resorption of this compound, a process common in animal systems. Supporting evidence for this also comes from the lack of significant induction of cell damage via loss of viability and lack of damage to the cellular membrane (LDH assay) even at high concentrations in the present study. This phenomenon has also been seen in other human in vitro intestinal models [55]. Given how close this trend is to intestinal responses in fish, it would be interesting to measure the metabolite formation in the medium and identify the degree to which transformation under in vitro conditions differs to results from in vivo or ex vivo methods (e.g. [40]).

Glucose absorption across the cells (lining the intestine) is the result of active transport, facilitated diffusion and metabolic utilisation. This glucose uptake is driven by the concentration gradient across the cell membrane. In this respect, low levels of glucose are typically present in the cytoplasm due to rapid phosphorylation by hexokinases, and so the transport will occur in the influx or uptake direction. Transepithelial transport is an energy consuming process, with the depletion of glucose indicative of increased cellular requirements. During

B[*a*]P exposure, limited glucose present at 24 h in all exposure solutions but especially medium is suggestive of an energy intensive process being undertaken. Indeed, the steep decline in glucose over the first 4 h suggests rapid uptake of this compound (glucose) from the medium into the cell for basic cellular maintenance. However, B[*a*]P uptake is likely a passive process and the suggestion that this toxic compound is potentially absorbed during this process may have some merit, given that the presence of a concentration gradient for B[*a*]P uptake has been previously observed in the catfish intestine [40, 56]. We postulate that at 24 h, the minimal presence of glucose in the medium/apical lumen would result in limited transport of B[*a*]P from the medium into the cells and should demonstrate a decrease in cytotoxicity and other biochemical parameters. Based on this supposition, it is unsurprising that cytotoxic activity (as measured by LDH and APH assays) is not significantly different over time (24 and 48 h), pH values, or concentrations, as limited uptake would have likely occurred via this pathway. This trend in the absence of cytotoxic activity following B[*a*]P exposure has also been observed in other in vitro models, such as the Caco-2 cell line [55] although the range of this study was substantially higher (1 - 300 μM). Prior studies in our laboratory have established that modification of the pH of the saline exposure solution can have a significant impact on the uptake of copper by the RTgutGC cell line [35], and we hypothesized that this trend would also occur under other exposure scenarios which were demonstrated in the current study. Interestingly, differences in glucose removal due to pH of exposure solutions identified differences between parallel pHs of 7.5 (medium) and 7.4 (L-15:medium). Previous research has reported that the transport of glucose is mainly affected by the amount of sodium in the intestinal lumen of rats and that the absorption of sodium is impaired at lower (7.0) and higher (8.5) pH [57]. With respect to the current study, minimal differences in sodium concentrations in the exposure matrix were observed (69 nM for L-15 medium and 44 nM for saline: medium respectively), suggesting that phenomenon may differ between experimental models. In human systems, the transport of water across the epithelial cells of the intestine has been attributed to osmotic gradients and sodium transport [58]. If we examine osmatic gradients in the exposure matrices, a difference emerges whereby 274 mOsm was recorded in the control matrix of L-15 medium and 204 mOsm in the saline: medium solution (irrespective of pH) suggesting that this may play an important role in the observed trend, but is now wholly responsible for the observed response. As such, we suggest that there may be a further component in this transport scenario given that if osmotic differences were the only driving force of this observed trend, then significant differences would have been observed between both saline: medium solutions rather than just one (pH 7.4).

In the present study, the potential of β-NF to induce, and α-NF to inhibit, P450 enzyme activity was assessed in the RTgutGC cell line using the EROD assay. The EROD assay, a broad marker for CYP1A families, requires the uptake, metabolism, and excretion of the substrate for any activity to be detected and as a consequence is suggestive of active CYP1A metabolism in any test system. In the current study, treatment of the cells with α-NF resulted in significant induction of EROD activity while co-exposure with α-NF led to an inhibition of EROD activity suggestive of the retention of active xenobiotic metabolism pathways in this in vitro model. In addition,

concentrations of the compounds required to induce this activity are comparatively minimal when compared to the literature. In microsomal fractions (S9) derived from the intestine of fish [40, 59, 60], a range of 0.4-40 pmol/min/mg protein has been reported, while in the primary culture of enterocytes of porcine origin, a basal value of 9 pmol/min/mg protein has been reported [61]. In contrast, liver microsomal fractions (S9) typically record a range of three times higher at a minimum with a range of <0.1-98 pmol/min/mg protein, irrespective of animal origin [62, 63]. In each instance, the concentration range required to induce EROD activity in microsomal fractions is quite variable (~1-100 μM β-NF) for intestinal cultures. Differences in basal EROD activity are not uncommon between or among fishes and several reasons related to the biology of fishes and physical environments can result in these differences [64]. It should be noted that when our study is compared to another human derived in vitro intestinal model (e.g. Caco-2; [55]), a response was elicited in terms of EROD activity at a much lower concentration (3.6 μM versus 50 μM) β-NF in the widely used Caco2 model. Indeed, this trend is also repeated in the inhibition of CYP1A following α-NF exposure, where EROD activity was inhibited using a dose of 200 μM α-NF (83% inhibition) [55]. In contrast, the current study recorded this trend using a dose of 100 μM α-NF which inhibited EROD activity in this cell line by 94%. Our data would suggest that this difference in basal EROD activity, previously observed between and among fishes, also extends to in vitro models and requires further investigations in order to identify differences in the biology and physical growth environments which cause these differences. In humans, the matrix in which PAHs such as B[a]P is transported has a significant influence across the intestinal epithelium barrier [65], and it could be argued that this would also hold true for exposures in other animal models. In vitro digestion models have been developed to assess the bioaccessibility of toxicants, such as PAHs, and account for pH variation in transport from the lumen to the enterocyte along the length of the intestine (incorporating three different intestinal regions) in humans. With respect to influence of pH variation for transport, the current study offers evidence to support its presence. Although a typical Gaussian or dose response curve was present for all exposure solutions, (*e.g.*, the data gives a maximum EROD response at a given exposure and drops thereafter), maximal induction varied between the two solutions irrespective of pH (medium versus medium: saline), with L-15 demonstrating the highest CYP1A induction following B[a]P exposure. As mentioned previously, the availability of transporters to uptake and transform B[a]P may vary between the two exposure solutions, in line with the variation in osmotic gradients and sodium concentrations. Further studies are required to elucidate the role of these confounding factors influencing transformation of B[a]P.

As previously mentioned, few studies have determined xenobiotic-conjugating enzymes in intestinal regions of fish. Consequently, less information is available for the presence of conjugating enzymes in the fish intestine [60]. To evaluate whether this intestinal in vitro model can carry out xenobiotic conjugation, total GST activity was assessed, using a commercial kit. Our data support the hypothesis that conjugation of B[a]P occurs, to a form that was more readily excreted into the medium (supported through the reduction of B[a]P in the medium after 48 h exposure). GST activity was found to differ between pH of solutions, but not over time or concentrations. As

previously observed, modification of the exposure pH in the RTgutGC model can mimic uptake rates of the mid and posterior intestine following exposure to copper, a ubiquitous environmental contaminant [35]. Due to limited data on intestinal metabolism enzymes in fish, extrapolation from human studies is necessary. GSTs are known to demonstrate patterns of expression similar to those of the P450s in small intestine, whereby expression levels decrease from the proximal intestine to the distal intestine. This trend in varying metabolic activity and transport rates between regions of the intestine has been suggested in rainbow trout for some metal contaminants [66–69]. In this respect, the data presented demonstrates the sensitivity and comparability of this cell line to in vivo observed responses. It should also be noted that although recordings of total GST activity in this RTgutGC cell line is equivalent to 44 ± 10 mol/min/mg protein (which is four times higher than GST data reported for catfish intestine [60]), this result is not surprising. In the analysis of biotransformation activities of the channel catfish *Ictalurus punctatus*, James *et al.* [60] reports on the cytostolic GST activity from microsome preparations of the intestine, rather than the total GST activity in the intestine. Microsomal preparations are unlikely to represent a full range of metabolism, in addition to potential species differences.

Genotoxic response is a cell-specific process, with toxicant exposure required to reach a threshold level before DNA repair systems are initiated. Studies which report on the induction of DNA damage following chronic and sub-lethal exposure to B[*a*]P are abundant in isolated organs [70–72] and cell lines [38, 73, 74]. Indeed, intestinal uptake and metabolism in the literature is well covered in both mammalian [74–76] and aquatic models [40, 77, 78] making this an ideal compound with which to establish the robustness of the fish based intestinal in vitro model. Toxic response is thought to occur primarily through the metabolic activation of PAHs to reactive intermediates and aryl hydrocarbon receptor (AHR)-dependent alterations in gene expression. Previous reports have seen significant differences in genotoxicity measured in blood cells (~2-22 % tail DNA) over a 50 d experimental period in rainbow trout [71]. In the current study, while % tail DNA ranged between 2-45% for different pH and exposure durations, significant differences were recorded at much lower concentrations compared to prior studies. Comparable trends for the induction were also observed for the EROD assay. Despite this causal relationship, no correlation existed between these two parameters (data not included). A large proportion of chemical carcinogens such as B[*a*]P require metabolic activation which are typically initiated by sequential reactions catalysed by P450 enzymes and epoxide hydrolase leading to the formation of the ultimate carcinogen, which subsequently interacts with the DNA of the target tissue to initiate damage (e.g. tumorigenesis). It is probable that a direct inhibition of P450 may lead to a decrease in enzyme activity and, as a consequence, decreased DNA damage, unless this inactivation results in a concurrent increase in ROS production. The decrease in DNA damage level as determined by % Tail DNA in the comet assay could also result from enhanced DNA adduct formation, if oxidative enzyme activity is enhanced or if activities of DNA repair enzymes are reduced. These could lead to hindered migration of DNA strands in the tail region. We did not measure DNA damage induced by ROS or adduct formation in the current study. The hypothesis that induction and suppression of CYP1A activity can result in oxidative stress is supported by the results of in vivo studies in the marine fish,

bird and reptile species exposed to the planar polychlorinated biphenyl (PCB) 3,3',4,4'-tetrachlorobiphenyl (TCB) [79–81]. It is encouraging to see a comparable in vitro to in vivo result highlighting the complexity of this intestinal culture system. Mechanisms of toxicity (with respect to B[*a*]P and other toxicants) with respect to dietary route of uptake, however, needs further elucidation.

In the present study, a low (2-8%) average % tail DNA for the untreated cells (negative controls) was observed. This observation is broadly in line with several studies carried out in our laboratory using other fish cell lines grown as monolayers [27–29]. There is however currently not consensus agreement among scientists regarding expected levels of DNA migration in control or untreated cells. OECD testing guidelines state that DNA migration in negative control cells should be as low as possible, preferably <6 % for mammalian liver cells under in vivo conditions [82], which is in line with observations made in the present study. In this context, OECD guidelines also suggest that detection of cross-linking agents is not the primary aim of the comet assay neither this assay is appropriate to detect aneugenic potential of chemicals [82]. It should, however, also be noted that the OECD guidelines do not mention control levels of DNA damage in cell lines used under in vitro conditions. Yet the guidelines do recommend that each laboratory have its own negative control historical data for specific experimental conditions. The levels of % tail DNA in the untreated single seeded cells in the present study is in line with our historical control (unpublished) data. This supports the OECD guidelines which suggest that the % tail DNA in negative controls should be within the pre-established laboratory background range for each individual tissue and sampling time [82].

Varieties of physio-chemical and biological factors are known to influence the induction of spontaneous levels of DNA damage as determined by the comet assay [25]. It is generally assumed that metabolic rate of fish cells (maintained at relatively reduced temperatures) is lower than mammalian cells [26]. As such, therefore, lower induction of spontaneous DNA damage in fish cells is not surprising. It is also possible that spontaneous levels of DNA damage are cell line-specific phenomena under specific experimental conditions. Although there is limited studies available, one mammalian study using the model Caco-2 cell line (derived from human colon carcinoma) also observed similar levels of induction in negative controls and a comparable range in treated cells when they were exposed to a PAH [73]. This supports the idea that the observed trend could be related to the tissue of origin for the cell line. The results of this study would suggest a need for the expansion and inclusion of recommendations for specific cells originating from different organs including for the comet assay [25]. Due to limited availability of intestine derived cell lines, it is also interesting to note the comparable observed trends between the two systems (i.e., fish and mammalian) and to postulate on the commonalities of organs performing similar functions. A prior study by Raisuddin and Jha [29] reported that fish cells were more sensitive compared to mammalian cells following exposure to environmentally relevant compounds. This sensitivity could however depend on the mechanisms of actions of the contaminants, the endpoints measured, the exposure duration and the protocol adopted [27].

In the aquatic environment, studies related to fundamental interaction between xenobiotic metabolism, transport and resultant toxicity in the intestine has been very limited. This is important not only for deep understanding but also to implement 3Rs principles. Some studies, however, do exist for B[*a*]P, which has allowed a comparative response between in vivo exposure and the in vitro model. Irrespective of the species, the intestine is a complex organ with varying degrees of metabolic activity throughout its length [78, 83, 84]. While cell lines offer some benefits, the complexity of the intestinal system requires careful cultivation to create a comparable and usable in vitro model. The present study highlights the existence of an active, highly efficient intestinal barrier against toxic dietary components and our evidence suggests the maintenance of cellular systems responsible for the metabolism of xenobiotics. More work is required to fill the knowledge gap including incorporation of transcriptomic and proteomics approaches that will enable a greater understanding of the preserved and active systems. Quantifying the degree to which the cell line response varies from the whole organism will hopefully provide weight of evidence in support of the use of this animal alternative model for chemical compounds with different mode or mechanisms of actions.

Overall, using the RTgutGC cell line as an in vitro model for fish intestine, we demonstrated that cultured enterocytes are capable of reducing the absorption of B[*a*]P via metabolism and suggest a redirected transportation pathway of metabolites back into the medium or lumen compartment. RTgutGC monolayers, which may be grown in a Transwell system as shown by us [35] to better mimic tissue complexity, represent a new and exciting tool to start probing mechanisms of toxicity and genotoxicity of a large suite of compounds and identify molecular initiating events (MIE) potentially allowing for reduction in the number of traditional animal tests. In the absence of any well validated model and lack of fish intestinal cell line, this model could potentially support a cost-effective, high-throughput, sensitive assessment of genotoxic potential for the contaminants which enter the body via the dietary route. Determination of the relative sensitivity of the RTgutGC cells, using a wide range of contaminants and endpoints with the well-established mammalian (i.e., Caco-2 cells) model will also help to further establish the usefulness of this fish model. This will provide insights of potential links between environmental and human health while supporting 3Rs principles.

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Figure legends and table captions

Fig. 1 Measurement of B[*a*]P present in culture medium and RTgutGC cells after 24 and 48 h of incubation in the dark (21C) at one dose, 0.2 μM B[*a*]P (determined from solubility in environment). Values are expressed as mean of the B[*a*]P corrected dose (accounting for adsorption to plastic ware) and standard deviation, with n=3 non-parallel passages. Inset into the figure is the autofluoresence of B[*a*]P (50 μM) in the RTgutGC cell line following a 24 h incubation. Scale bar is 20 μm.

Fig. 2 Glucose measured in medium (pH 7.4) demonstrating a declining trend over time irrespective of B[*a*]P concentrations.

Fig. 3 Ethoxyresorufin-O-deethylase (EROD) activity in RTgutGC cells grown and exposed in L-15 medium with 0.1 % DMSO to a range of B[*a*]P. Data is expressed as pmol of resorufin per min per mg protein and is representative of three individual experiments (non-parallel passages). No significant differences were observed between concentrations on transformed data, but significant differences were observed between the two sampling time points ($p < 0.001$).

Fig. 4 Glutathione transferase (GST) activities in the RTgutGC cell line at pH 7.5, 7.7, and 7.4 after 24 h exposure. No significant differences were observed between concentrations or time but where observed between pH's of exposure solutions ($p < 0.05$).

Fig. 5 Genotoxic response of the RTgutGC cell line to concentrations of B[*a*]P in two different media at three different pH values. Significant differences were found between 24 h (a) and 48 h (b) and between the solvent controls and the upper B[*a*]P concentrations (*** is equivalent to a $p < 0.001$). No significant difference was found between the pH values.

Table 1 Rate of glucose depletion as determined from the medium of the RTgutGC cells over a 24 h period. Rates are calculated relative to the decrease in μg glucose in the medium from the initial sampling point at 0 h up to 24 h. Data is presented as the mean ± standard error of the mean due to unequal sample size. As can clearly be seen, in both normal medium pH (7.5) and saline pH 7.7, glucose is almost completely depleted after 24 h.

Table 2 Mean EROD activity with standard error values (due to uneven sample sizes) of ethoxyresorufin-O-deethylase (EROD) levels in the RTgutGC cell line cultured in L-15 medium and exposed to 0.1% DMSO in L-15 medium (solvent control), β-naphthoflavone (CYP1A inducer), α-naphthoflavone (CYP1A inhibitor) or β-

naphthoflavone + α-naphthoflavone. Concentrations of α-naphthoflavone chosen to inhibit CYP1A activity were derived from James *et al.* [78].