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Improvements in single-use bioreactor film material composition leads to robust and reliable Chinese hamster ovary cell performance

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Abstract

Single-use technologies, in particular disposable bioreactor bags, have become integral within the biopharmaceutical community. However, safety concerns arose upon the identification of toxic leachable compounds derived from the plastic materials. Although the leachable bis(2,4-di-tert-butylphenyl)-phosphate (bDtBPP) has been previously shown to inhibit CHO cell growth, it is critical to determine if other compounds like this are still present in subsequent generations of films for industrial application. This study compares the performance of CHO cells, CHO-K1 and CHO-DP12, cultured in media conditioned in an older single-use bioreactor (SUB) film (F-1) and a newer generation film (F-2) from the same vendor. CHO cells cultured in media conditioned for 7 days in the F-1 film demonstrated significantly reduced growth and antibody productivity profiles when compared to controls and media conditioned for the same time period in the newer F-2 film. Proteomic profiling of CHO cells cultured in the F-1 conditioned media identified differentially expressed proteins involved in oxidative stress response as well as compromised ATP synthesis. These potentially metabolically compromised cells exhibited reduced oxidative phosphorylation activity as well as lower glycolytic metabolism, characteristic of slower growing cells. Non-volatile and metal leachables analysis of film extracts by LC-MS revealed a reduction in the abundance of the analysed leachates from F-2 films when compared to F-1 films including bDtBPP, potentially explaining improved CHO cell growth in F-2 conditioned media. Furthermore, *in vitro* endocrine disruptor testing of the known leachable revealed this molecule to possess the potential to act as an androgen antagonist. This study demonstrates an improvement in the materials composition used in modern generations of SUBs for safe application in the bioprocess.

Keywords: Single-use bioreactors, Leachables, Chinese hamster ovary cells, Endocrine disruptor, bDtBPP, Mitochondria, SeaHorse XF96

1. Introduction

With the emergence of novel bio-therapeutics, the advent of bio-similars and the increasing number of lead molecules within the developmental pipelines, the notion of individual manufacturing sites being constructed at a cost of ~€1 billion for every newly approved product is not sustainable [1]. Increasing the flexibility of these manufacturing sites leading to the introduction of multi-drug facilities has been accomplished in certain cases through the implementation and integration of single-use disposable technology in place of or in parallel with existing stainless steel, hard-piped production platforms. Applying single-use technology to the bioprocessing pipeline carries with it a cohort of potential benefits including reduced CapEx of up to 75%, elimination of clean-in-place/steam-in-place treatments, thereby reducing operational costs, as well as minimising the environmental carbon footprint [2,3]. Although the size limitations of SUBs (2,500L) does not compete with their stainless steel bioreactor counterparts (10-25k L), continuous bioprocessing, perfusion-based continuous bioproduction, upstream seed trains and mixed-use facilities in addition to buffer/media holding are all key advantages supported by single-use systems [4].

The Chinese hamster ovary (CHO) cell is the predominant cell line used in the production of recombinant therapeutic proteins for the last 3 decades [5]. Advancements in the areas of cell line development, bioprocess design and media composition has allowed for the multi-gram/L titres that we routinely see today, opposed to the mg/L of the 1980s. Pragmatically, these low titres have contributed to the requirement of large-scale fermenters that remain the standard unit of operation today. Despite the limitation in SUB size, further boosting the g/L titres from CHO cultures could mediate similar outputs from SUB batch cultures compared to large-scale SS bioreactors thereby potentially making the implementation of these scale-down systems more tangible [6]. Of course with novel and more bespoke biologics being developed, difficult-to-express proteins are a constant challenge to the industry and remain a driving force for the generation of more sophisticated production CHO cell lines via approaches such as microRNA engineering [7], genetic knockouts [8], removing unnecessary genomic burdens [9] and enhancing promoter performance [10].

One major concern associated with SU technology within the biomanufacturing space is the lack of clarity surrounding the leachable profiling from these predominantly polyethylene-based plastics. Previous reports with regards to the interaction of these plastics with media components such as cholesterol adsorption resulting in inhibited NS0 cell growth [11] as well as

chemical transformations introducing potentially toxic leachables into the culture media raises further concerns relating to any new material entering the bioprocessing pipeline. Routinely, additives are required for efficient processing and manufacturing of the plastic films such as the secondary anti-oxidant Irgafos® 168 which upon gamma-irradiation becomes oxidised and subsequently breaks down to leach degradation products into the culture media [12]. The optimal culture environment within the bioreactor is critical to CHO cell performance and can be compromised by these leachable compounds that can be potentially toxic. For example, bis(2,4-di-*tert*-butylphenyl)-phosphate or bDtBPP was initially reported by Hammond and colleagues in 2013 [13]. Originating from the secondary anti-oxidant Irgafos® 168 or tris(2,4-di-*tert*-butylphenyl)-phosphite, bDtBPP was reported to inhibit cell growth in a variety of proprietary CHO cell lines at concentrations ranging from 0.12-0.73 mg/L [14]. More recently, studies from our group found this leachate to be toxic at far lower concentrations than previously reported (0.035-0.1 mg/L) [15] which fall within the concentration range that has been observed to leach and accumulate under ambient conditions over a time period of 3-4 days (0.025-0.11 mg/L) [14].

Leachable components such as bDtBPP have been reported to negatively impact cell culture performance not only in terms of growth/viability, but also titre or product quality [16]. Although it would be advantageous to know the material composition so that potential causes may be evaluated, this information is usually not completely available from manufacturers. Also, due to the complexity of the bag manufacturing process, it is difficult to determine the exact composition of most bag films. However, it is known that degradation of the polymers and additives in SUBs occur most significantly during high-energy processes, namely sterilization [17] and film extrusion [18], which may produce degradation products such as bDtBPP, whose potential effects on cells maybe completely overlooked. This reality, combined with the low concentrations reported to elicit negative effects on cell growth makes it paramount that the appropriate solvent systems and analytical techniques for extractables and leachables (E&Ls) screening are being implemented that are sensitive enough to detect both low abundant leachates as well as capturing all leachables present [19,20].

Increased understanding and characterisation of both the leachables profile from SUBs and their effects on cells is needed. At present, there are some standardised cell culture tests available [21,22] to assess the biocompatibility and safety of production cell lines [22,23], which will assist both bag manufacturers in developing and qualifying new bag films, and end-users to proactively select bags to be used in their processes. However, considering that cell lines show

different sensitivities to leachables components [22], developing widely applicable cell culture tests is not a trivial exercise, but it would, however, be desirable, so that vendors and users can apply them with confidence and avoid unwanted false-positive results [23].

In addition, besides cell culture tests, plastic films used in biomanufacturing should also report leachables assessments in order to be considered fully characterised for their compatibility and safety in relation to cell culture performance as the cocktail of potential leachates other than bDtBPP could pose a problem. Although there is now a greater awareness of the use of single-use components in biomanufacturing, a comprehensive characterisation at all levels, both biological and chemical, should be an inevitable requirement for these SUBs to be available in the market. A large-scale assessment of E&Ls from 34 single-use plastic films used in cell cultivation has highlighted that the spectrum of E&Ls has changed due to the introduction of new types of polymers as well as manufacturing changes [20], whose potential biological effects should be addressed. In view of this, new regulations and standardised guidelines should be established by official institutions such as the Food and Drug Administration (FDA) and United States Pharmacopeia (USP), working in collaboration with industry groups such as BioProcess Systems Alliance (BPSA), the Parenteral Drug Association (PDA), the Product Quality Research Institute (PQRI), Dechema and Biophorum Operation Group (BPOG) [24].

In this study we performed a comprehensive examination of the impact of newer materials on both the leachables profile as well as the influence these materials had on CHO cell growth. Two generations of single-use films from the same vendor were chosen, a newer generation film (F-2) that was designed with lower levels of Irgafos® 168 compared to one of its predecessors (F-1). This strategy used by bag manufacturers would ultimately reduce the amount of the toxic bDtBPP that could potentially leach into the contacting media. Culture media was conditioned in each film as a means to capture the leachates derived from each plastic with subsequent growth profiling of two CHO cell lines. Leachable profiling of these two films in conjunction with cell-based assays using the same conditioned media afforded the ability to directly compare cellular performance and leachables content with particular emphasis on changes within the identified leachates, keeping known toxic leachables like bDtBPP in mind. This work also provides information about other sensitive tests that might be included with a view to the development of standardised culture cell tests that would enable the early identification of non-satisfactory films for cultivation of CHO cell lines in chemically defined media. We also demonstrate that leachables can potentially act as endocrine disruptors.

2. Materials and Methods

2.1 Single-use bioreactors (SUBs) used in this study

Materials from two model generations of the same brand of SUBs (F-1 and F-2) were evaluated. These were multilayer films containing linear low density polyethylene as the fluid contact layer and ethylene-vinyl acetate as the gas barrier and outer layers. The model F-1 is an old version of this film while F-2 is a more recent version.

2.2 Conditioned media generation

F-1 and F-2 SUBs (10 L total volume) were incubated with 2 L of the chemically defined BalanCD® CHO Growth A media (Irvine Scientific®) in the presence of 1% penicillin/streptomycin solution (Sigma–Aldrich). Sealed bioreactors were incubated on a Biostat® rocking motion incubator (Sartorius); temperature was set at 37 °C and the incubation was carried out for a duration of 3 or 7 days. To ensure good mixing and complete contact with bioreactors films, no inflation was used during the incubation and optimum speed and max rotation angle were used thus minimizing foam formation. Negative controls were generated, incubating 2 L of media in the presence of 1% penicillin/streptomycin in pre-rinsed and autoclaved grade A glass bottles, using the same conditions for incubation. Additionally, the same tubing was used to fill the bags as was used to fill the bottles for consistence purposes. Incubation with SUBs was performed in triplicate using bags from the same production lot. Negative controls were generated in triplicate as well. At the end of incubation time, all media samples were stored in the original plastic bottles at 4 °C. The labile component L-glutamine (Thermo Fisher Scientific) was added to the media after conditioning and just prior to setting up cell cultures.

2.3 Cell Culture Conditions and Growth/toxicity assays

A parental non-producing CHO-K1 (ATCC® CCL-61™) and an IgG-producing CHO-DP12 (ATCC® CRL-12445™ Clone #1934) cell line was routinely cultured in chemically defined media, BalanCD CHO Growth A, supplemented with 4 mM L-Glutamine and the anti-clumping agent 2% polyvinyl alcohol. CHO-DP12 cells were maintained stable by pulsing, every 3 passages, with 200 nM of methotrexate (Sigma-Aldrich). Cell assays were inoculated at a starting cell density of 2 x 10^5 cells/mL in a 5 mL volume in a 50 mL filtered-topped tube

(Helena BioSciences Europe) and maintained under ambient culture conditions of 37°C, 5% CO_2 and 170 rpm. Viable cell density and cellular viability was assessed using a benchtop flow cytometer, Guava EasyCyte 5HT system (Millipore, Billerica, MA) in combination with the ViaCount assay (Millipore) as per manufacturer's specifications. Additionally, metal ion screening was performed using the following metal salts; Lead Nitrate, Silver Nitrate, Nickle(II) Chloride Hexadydrate, Sodium Molybdate dihydrate and Aluminium Chloride (Sigma-Aldrich), all dissolved in water and screened at a concentration of 1 mg/mL based on the molecular weight of the metal and not the salt.

2.4 ELISA – IgG productivity assay

CHO-DP12 culture supernatants were collected by centrifugation at 1,000 x g for 5 min, the IgG containing supernatant was decanted and samples were diluted appropriately for each time point in diluent buffer. The ELISA work-flow was carried out in accordance with the manufacturer's specifications (Bethyl Laboratories).

2.5 Leachable testing on conditioned media

Non-targeted analyses were performed for identification of non-volatile leachables by LC-MS. Extracts were treated using DLLME [25] to remove surfactants and other matrix components present in the media that could cause ion suppression. HRAM full-scan MS analyses was performed on a Thermo Scientific™ UltiMate™ 3000 RS coupled to a Thermo Scientific™ Q Exactive™ Plus mass spectrometer with HESI-II interface [20]. Resulting HRAM data were processed with Compound Discoverer™ 2.0, followed by automatic online library search against mzCloud.org advanced mass spectral database (HighChem) and ChemSpider (Royal Society of Chemistry), as well as a local E&L compound database. Mass Frontier 7.0™ software was used to determine most probable structures if several options for components were returned. For each proposed structure, the 'Fragments and Mechanisms' feature in Mass Frontier was used to generate predicted 'fragments and mechanisms' through the HighChem Fragmentation Library™ searching. A high degree of correlation between predicted and experimental fragments was required to confirm the proposed structure.

A multi-element determination was also performed on the extracts by ICP-MS. Extracts were acidified with a mixture of 2% (v/v) HNO_3 and 0.5% (v/v) HCl, in 1:9 sample/acid (v/v) prior

to analysis. All 30 analytes, ^{75}As, ^{111}Cd, ^{202}Hg, ^{208}Pb, ^{7}Li, ^{52}Cr, ^{60}Ni, ^{63}Cu, ^{118}Sn, ^{121}Sb, ^{137}Ba, ^{27}Al, ^{55}Mn, ^{56}Fe, ^{66}Zn, ^{51}V, ^{59}Co, ^{78}Se, ^{95}Mo, ^{101}Ru, ^{103}Rh, ^{105}Pd, ^{107}Ag, ^{189}Os, ^{193}Ir, ^{195}Pt, ^{197}Au, ^{205}Tl, ^{88}Sr, and ^{209}Bi, were measured with a Thermo Scientific™ iCAP™ RQ ICP-MS, according to a previous study [20]. Elemental concentration was determined using calibration curves from multi-elemental standards.

2.6 Endocrine disruptor testing of bDtBPP

2.6.1 Cell culture

All cell lines were grown in 75 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) at 37°C with 5% CO_2 and 95% humidity. The human adrenal carcinoma (H295R, ATCC) cell line was routinely cultured in H295R cell culture medium containing Dulbecco's modified Eagle medium (DMEM, Life Technologies) with Ham's F-12 nutrient mixture (1:1) supplemented with 1% ITS + Premix (BD Biosciences) and 2.5% NuSerum (BD Biosciences). The H295R cells were seeded in H295R assay media composed of DMEM with Ham's F-12 nutrient mixture (1:1) supplemented with 1% ITS + Premix and 2.5% charcoal stripped serum (Sigma-Aldrich).

2.6.2 Reporter gene assays (RGA's)

Four reporter gene cell lines were previously developed by transforming human mammary gland cell lines with the luciferase gene under the control of a steroid hormone inducible promotor as described in Willemsen et al [26]. The TARM-Luc cell line is specific for the detection of androgens and progestagens, MMV-Luc cell line for oestrogens, TM-Luc for progestagens and TGRM-Luc for glucocorticoids and progestogens [27]. These transformed cell lines were routinely cultured in cell culture medium containing DMEM and 10% foetal bovine serum. DMEM without phenol red was used when culturing the MMV-Luc cell line. RGA's were carried out in assay media composed of DMEM and 10% hormone depleted serum as previously described by Frizzell et al. [28]. Briefly, cells were seeded at a concentration of 4×10^5 cells/mL,100 μL/well into white walled, clear and flat bottomed 96-well plates (Greiner, Bio-One, Frickenhausen, Germany) and incubated for 24 hours at 37 ºC with 5% CO_2 and 95% humidity.

After 24 hours, bDtBPP and the relevant steroid hormone standards dissolved in DMSO was added to the cells at a final DMSO concentration of 0.1%. The final concentrations of bDtBPP during cell exposure was 0.005, 0.025, 0.035, 0.05, 0.1 and 0.25 µg/mL. The positive controls were: 1.36 ng/mL 17b-estradiol (MMV-Luc), 14.5 ng/ml testosterone (TARM-Luc), 157 ng/mL progesterone (TM-Luc) and 181 ng/mL cortisol (TGRM-Luc). A solvent control was also included for each cell line (0.1% v:v DMSO in media). Antagonist tests were carried out by incubating bDtBPP (0.005, 0.025, 0.035, 0.05, 0.1 and 0.25 µg/mL) with the relevant positive control for the cell line being tested. The cells were incubated for 48 h, the supernatant discarded and the cells washed once with 200 µl PBS. The cells were lysed by adding 25 µL cell lysis buffer (Promega, Southampton, UK) to each well. Finally, 100 µl luciferase substrate (Promega, Southampton, UK) was injected into each well. Luciferase activity was measured using a Mithras Multimode Reader (Berthold, Other, Germany) and the response compared to that of the solvent and positive controls. RGAs were performed in triplicate for each experimental point and in three independent exposures. The MTT cell viability assay was also performed on the treated RGA cells in parallel to this assay.

2.6.3 H295R steroidogenesis assay

The H295R cell line was obtained from the American Type Culture Collection LGC Standards. The assay was carried out as described previously [29]. Briefly, the H295R cells were seeded at a concentration of 3 x 10^5 cells/mL, 1 mL/well, into 24-well cell culture plates (BD Biosciences, Bedford, MA, USA). The cells were allowed to attach for 24 hours. Following cell attachment, the media was replaced with fresh H295R assay media containing the test compounds **(**bDtBPP 0.005, 0.025, 0.035, 0.05, 0.1 and 0.25 µg/mL) dissolved in DMSO at a final concentration of 0.1% (v:v). Forskolin 10 µM (FSK10) was used as a positive control and 0.1%, v: v DMSO in media as a solvent control in triplicate. Forskolin has been shown to act as general inducer of steroidogenesis in the H295R cell line via activation of cAMP pathways, resulting in elevated levels of oestradiol, testosterone and progesterone [29]. After 48 hours of exposure, media was collected from each well and stored at -20°C until hormone quantification was carried out using highly specific ELISAs as outlined previously [27]. All experimental points in the H295R assay were performed in triplicate and repeated in three independent exposures. The AlamarBlue® cell viability assay was performed on the cells remaining in each well.

2.6.4 Hormone detection and quantification

The steroid hormones oestradiol, testosterone, progesterone and cortisol levels in the media were quantified in duplicate by highly specific ELISA assays (DRG Diagnostics, Marburg, Germany). These highly specific kits are based on the principle of competitive binding and are intended for the quantitative *in vitro* diagnostic measurement of oestradiol (0-2000 pg/mL), testosterone (0-16 ng/mL), progesterone (0-40 ng/mL) and cortisol (0-800 ng/mL), with sensitivities of 10.60 pg/mL, 0.083 ng/mL, 0.045 ng/mL and 2.5 ng/mL respectively (DRG Diagnostics, 2016; DRG Diagnostics, 2009; DRG Diagnostics, 2007; DRG Diagnostics, 2006). In order to assess their suitability for measuring the steroid hormones, oestradiol, testosterone, progesterone and cortisol standard curves were prepared in H295R cell cultures. The ELISA was validated for measuring the steroid hormones in this culture medium. All other steps in the ELISAs were carried out in accordance with the manufacturer's instructions (DRG Diagnostics, 2016; DRG Diagnostics, 2009; DRG Diagnostics, 2007; DRG Diagnostics, 2006). The intra-assay coefficients of variation were less than 10%. Each ELISA plate contained a standard curve, solvent control, positive control and the test compound bDtBPP concentrations. The optical density was measured at 450 nm wavelength using a Sunrise spectrophotometer (TECAN, Switzerland). The mean absorbance obtained from each standard was plotted against its concentration using dose-response curves generated with GraphPad PRISM 5 software.

2.6.5 Cell viability assays

As well as visual inspection of the RGA and H295R cells under the microscope to evaluate cell morphology and attachment, cell viability assays were performed in parallel to the assays to check for any toxic effects of the concentrations of bDtBPP which the various cell lines were exposed to. For the H295R and RGA cell lines, the AlamarBlue® assay [30] and MTT assay [31], respectively, was carried out as reported previously.

2.6.6 Statistical analysis

All experimental points in the H295R assay, RGAs and cell viability assays were performed in triplicate and repeated in three independent exposures. All values shown are expressed as mean ± standard error of the mean of the three independent exposures (n=3). Data were analysed using Microsoft Excel and Graphpad PRISM 5.01 software. A one way analysis of variance

(ANOVA) and Dunnett's Multiple Comparison Test was used to determine significant differences between the treatments and the corresponding controls. The mean concentrations were tested for significant difference at the 95% confidence level. The criterion for significance was considered as *p*-value of ≤ 0.05, $p = \leq 0.05$ (*) ≤ 0.01 (**) ≤ 0.001 (***).

2.7 Metabolic profiling using the SeaHorse Agilent Seahorse XF-96 extracellular Flux Analyser

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the cell metabolic analyser Seahorse XF96 (Agilent technologies) according to the manufacturer's instructions. $2x10^5$ cells were cultured in 5 mL of media conditioned in F-1, F-2 and Control glass bottles and cultured for 96 hours at 37 ºC. A Seahorse XF96 cell culture plate was previously coated with Corning Cell-Tak cell and tissue adhesive (Corning™, 22.4 µg/mL) and incubated for 1 hour in a 37 ºC non-CO_2 incubator. Conditioned media were replaced by unbuffered Seahorse XF media for Phenotype assays and with Buffered Seahorse XF media (without phenol red) for glycolytic rate assays. In both cases, Seahorse XF media were supplemented with 10 mM glucose, 1 mM pyruvate and 2 mM glutamine and pH adjusted to 7.4. Finally, 2 x 10^4 viable cells/well were pelleted, re-suspended in the corresponding Seahorse XF media and plated into the previously coated plate (50uL containing 400 cells/µL per well, 2 x 10^4 viable cells/well). Plates were centrifuged at 300 g for 1 minute, incubated at 37 °C in a CO_2-free incubator for 30 min and 150 µL of Seahorse media were carefully added to each well, followed by a 30 minute incubation. Three real-time measurements of OCR and ECAR were directly measured (basal readings) and after injection of different mitochondrial inhibitors or glycolytic modulators. For phenotypic assays, the cells were treated with optimized concentrations of oligomycin (1 µM), carbonyl cyanide p-[trifluoro-methoxy]-phenyl-hydrazone (FCCP; 1 µM). For the glycolytic rate assays the cells were instead sequentially treated with optimized concentration of rotenone + Antimycin A (0.5 µM) and 2-deoxy-D-glucose (50 mM). Oligomycin, FCCP, rotenone + antimycin A and 2-deoxy-D-glucose concentrations were optimized according to the manufacturer's instructions. The corresponding assay, phenotype assay or glycolytic rate assay were run using the Seahorse XF-96 analyser pre-programmed templates for these assays.

352 *2.8 Quantitative Label-free LC-MS/MS Proteomic Analysis*

353 Cell pellets from the CHO-K1 and CHO-DP12 cell lines treated with BalanCD® Growth A
354 media conditioned in both F-1 and F-2 films for 7 days as well as negative control media were
355 lysed in a buffer containing 7 M urea, 2M Thiourea, 4% CHAPS and 30 mM Tris at pH 8.5.
356 Protein concentration was determined using a QuickStart Bradford protein assay (Biorad) and
357 100 µg of each sample was prepared for quantitative proteomic analysis. Protein samples were
358 prepared as previously described using the Filter Aided Sample Prep (FASP) method and C18
359 peptide purification [32,33]. Nano LC-MS/MS was performed using a Dionex Ultimate 3000
360 nanoRSLC (Thermo Scientific) coupled in-line to an Orbitrap Fusion Tribrid mass
361 spectrometer (Thermo Scientific). LC-MS/MS methods were applied as previously described
362 [34]. Protein identification was achieved using Proteome Discoverer 2.2 with Sequest HT and
363 MASCOT algorithms. Data was searched against the NCBI Chinese Hamster Ovary
364 (*Cricetulus griseus*) protein database downloaded in November 2017 containing 24,906
365 sequences. Quantitative label-free data analysis was performed using Progenesis QI for
366 Proteomics (version 2.0, Nonlinear Dynamics, Waters) essentially as described by the
367 manufacturer. Protein identifications from Proteome Discoverer were imported into Progenesis
368 QI for Proteomics for differentially expressed proteins. Proteins were considered differentially
369 expressed if they passed the following criteria (i) a minimum of 2 peptides contributing to a
370 protein identification (ii) an ANOVA *p*-value score <0.05 between sample sets and (iii) a
371 minimum of 1.5-fold change in abundance between sample sets.

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Results

Cell compatibility testing of film conditioned growth media

We performed biocompatibility profiling of two films sourced from the same vendor; one of which was an older film (F-1) and one a more modern film (F-2) of the same design. To assess the potential cellular toxicity of leachable compounds derived from newly developed materials as opposed to single molecule screening, as in the case bDtBPP [15], we conditioned chemically defined media using SUBs made of the two referred films, F-1 and F-2. The cocktail of other leachates and the array of concentrations that each compound accumulates, could all play a role in toxicity. These two films were chosen to determine, in an extreme case of a poor performing SUB versus a good performing SUB, what the influence to CHO cell cultures was.

In keeping with our previous study [15], both the parental CHO-K1 and the monoclonal antibody (mAb)-producing CHO-DP12 cell lines were evaluated for compatibility with both film-conditioned media. Culture media was conditioned in both F-1 and F-2 films over a 7 day time course to capture the full complement of potential plastic sub-components to leach into the culture media and to do so at concentrations most likely to elicit toxic effects. Following on from this, the same basal culture media was carried through an identical conditioning process in both F-1 and F-2 films for a 3 day time course. This more modest conditioning time was selected to reflect both previously published conditioning timelines [14] as well as to determine the potential toxicity to cultures in the immediate stages of culture.

CHO-K1 cells cultured in media conditioned for 7 days in the F-1 film demonstrated considerably reduced growth when compared to control cultures (**Fig. 1A**). This growth inhibitory F-1 media did not appear to negatively impact CHO cell viability (**Fig. 1B**). In contrast, CHO-K1 cells cultured in F-2 film conditioned media exhibited no adverse growth effects when compared to controls (**Fig. 1C** and **D**). Similarly, when the mAb-producing CHO-DP12 cell line was subjected to the same 7 day conditioned media, the F-1 film was observed to reduce cell growth with no deleterious effects on culture viability when compared to controls (**Fig. 1E** and **F**). A ~60% reduction in mAb production (**Fig. 1G**) was observed in the case of F-1 cultured CHO-DP12 cells. As in the case of CHO-K1s, CHO-DP12 cells cultured in the F-2 conditioned media behaved similar to the controls while maintaining similar antibody productivity when cultured in the 7-day conditioned media (**Fig. 1 H-J**).

We next generated conditioned media using both F-1 and F-2 films for a time course of 3 days. CHO-K1 cells cultured in conditioned media from both F-1 and F-2 films demonstrated

comparable growth and viability to control media (**Fig. 2A-D**). In the case of CHO-DP12 cells, cell growth, viability and productivity remained similar to that of controls for both F-1 (**Fig. 2E-G**) and F-2 (**Fig. 2H-J**) films.

Label-free LC-MS/MS profiling of the toxic versus compatible films

From the cell toxicity data, it is evident that conditioned media generated from the F-1 film over a 7-day time course is inhibitory to CHO cell growth and productivity when compared to a more modern film conditioned for the same time. For this reason, we performed label-free LC-MS/MS proteomic profiling on CHO-K1 and CHO-DP12 cells cultured in both films conditioned for 7 days only.

Label-free LC-MS/MS on CHO-DP12 and CHO-K1 cells cultured in the toxic F-1 conditioned media and sampled after 96 hours revealed 155 differentially expressed (DE) proteins in both CHO-DP12 and CHO-K1 cells (**Sup. Table 1**) with 35 common across both cell lines (**Fig. 3A**). Qualitatively, 4667 and 5092 proteins were identified in CHO-DP12 and CHO-K1 cells, respectively (**Sup. Table 2**), cultured in F-1 conditioned media with **Table 1** showing a subset of the common DE proteins between both cell types.

One interesting group of proteins that were down-regulated in both CHO-DP12 and CHO-K1 cells exposed to F-1 film conditioned media were all components of the NADH dehydrogenase enzyme (Complex I), *NDUFS2*, *NDUFS8*, *NDUFV1*, and *NDUFA10* of the mitochondrial electron transport system (ETS). Given this observation, we assessed mitochondrial function through the measurement of oxygen consumption rate (ECR) using a micro-plate based measurement (Seahorse Bioscience XF Analyzer). The rate of oxygen consumption was reduced in CHO cells cultured in F-1 conditioned media where as normal mitochondrial activity was observed in cells cultured in F-2 conditioned media when compared to negative controls (**Fig. 3B**). When extracellular acidification rate (ECAR) of the culture media was determined, it was observed that slow growing CHO cultures from F-1 conditioned media exhibited a reduced rate of extracellular media acidification when compared to F-2 conditioned media and negative controls (**Fig. 3C**). Gene-Ontology analysis based on DE proteins in CHO-K1 and CHO-DP12 cells cultured in F-1 film conditioned media revealed the enrichment for biological processes relating to oxidative stress and mitochondrial respiratory chain complexes (**Table 2**). Up-regulation of the mitochondrial Lon Protease (*LONP1*) was identified in F-1 cultured CHO cells which is a protein responsible for responding to acute oxidative stress [35].

When CHO-DP12 and CHO-K1 cells cultured in the non-toxic F-2 film conditioned media and subjected to proteomic analysis, it was interesting to observe 108 and 268 proteins DE in CHO-DP12 and CHO-K1 cells, respectively (**Sup. Table 3**). There were 19 DE proteins common to both cell types with two proteins demonstrating contrary expression patterns, glutathione synthetase and coatomer subunit alpha. When compared to the 35 common DE proteins from cells cultured in the toxic F-1 conditioned media (**Table 1**), there was 1 protein found to overlap between the non-toxic F-2 film and the toxic F-1 film, transferrin receptor protein 1 (*TFCR*), (**Fig. 3A**). However, although this common DE protein was found to be decreased in its expression in cells cultured in the toxic F-1 conditioned media, it was highest in its abundance in CHO-DP12 and CHO-K1 cells cultured in F-2 conditioned media. It is apparent from this study that media conditioned from a SUB film (F-2) that does not impact on CHO cell growth does still influence the cells proteome. It is not surprising that differential protein expression was observed for both films as leachables were present in both cases as the following analysis will demonstrate.

Leachable profiling of conditioned media

Sixteen compounds with confirmed and confident identification from the media extracts by LC-MS are listed in **Table 3**. Twelve of the 16 compounds (75%) were present at higher levels in F-1 bags compared to F-2 (**Fig. 4A**), including 2 degradation products of Irgafos®168: bDtBPP, which has negative effects on CHO cells, as indicated in previous sections, and Irgafos® 168 oxidized form. The non-volatile compounds N,N-dimethyldecan-1-amine ($C_{12}H_{27}N$) and *cis*-1,3-docosenic acid amide (Kemamide® E ultra) ($C_{22}H_{43}NO$) were detected only in F-2 bags. **Figure 4B and C** shows LC chromatograms for F-1 and F-2 extracts, where some leachables with higher concentrations in F-1 are highlighted, including bDtBPP as mentioned previously.

In general terms, the identified non-volatile compounds belong to different chemical classes and also perform different functions as components of plastic materials. Major extractable compounds identified can be classified as antioxidants and their degradation products, plasticisers, polymer-related compounds, as degradation products and building blocks, and residues from the polymerisation process, such as initiators and catalysts. The groups of compounds with specific functions that were identified in the samples are also reported in

Table 3. Several of these compounds were already reported previously [36–38]. Intact Irgafos®168 was not detected under any extraction condition.

The identified compounds were also structurally classified via Toxtree version 2.6.0 [39] using the Cramer rules with extensions. Based on quantitative structure–activity relationships (QSARs), the Cramer classification is a rules-based process that sorts compounds into three classes; Class 1 (low risk of toxicity), Class 2 (intermediate between 1 and 3), and Class 3 (either no basis to presume safety or suggest significant toxicity). Additionally, the entire population of compounds was assessed by *in silico* QSAR analysis for their mutagenic/carcinogenic potential using the Benigni/Bossa rule base. The Cramer classifications and mutagenicity assessments were performed using the appropriate modules of Toxtree software. Results indicated that most leachables (75%) are quite toxic, being classified in Cramer Class 3 and only 25% were in Cramer Class 1. But nevertheless only 2 compounds (13%) triggered an *in silico* alert for mutagenicity, which also belong to Cramer Class 3. The referred compounds, 2,(4 or 6)-toluendiamine and 2,4-dimethyl-aniline, are residues from the polymerization process, and have presumably the highest safety risks of the group. Additionally, these compounds are also at higher levels in F-1 bags compared to F-2.

Regarding elemental analysis, only 9 elements of 30 were found in the samples (**Table 3**). As shown in **Figure 4D**, 4 of the 9 elements (44%): Ni, Ag, Sn, and Ba were at higher concentrations in F-1 bags, and the other 5 elements (56%): V, Mo, Cd, and Pb, showed the highest levels in F-2 bags, while Cu levels were similar in both SUBs. V and Cd were found only in F-2 bags, while Ba was only found in F-1 bags. The ICH Q3D guidelines [40] classify elements in three classes based on their toxicity and likelihood of occurrence in the drug product, and also specify both daily doses and concentration limits of metallic impurities in pharmaceutical final products and in active pharmaceutical ingredients and excipients. According to these guidelines, the highest levels of Pb and Cd, Class 1 elements and the most toxic ones were found in F-2 films, followed by Mo and V that belong to Class 2A. Ag, also a toxic element (Class 2B) is the only element of concern that was found at higher concentrations in F-1 films (**Fig. 4E** and **Table 3**).

Interestingly, when a subset of metal ions (Ag, Pb and Ni) were screened in CHO-K1 and CHO-DP12 cells at a high concentration of 1 mg/L, similar to previous screening studies [13,15], Ag was found to be the only metal to elicit toxic effects on growth of both CHO-K1 and CHO-DP12 cells (**Fig. 5A** and **C**). Silver (Ag), previously categorised as a toxic element (Class 2B)

was observed to be at a higher abundance in F-1 films than F-2 films (**Fig. 4D**). However, when lower concentrations of Ag were screened, the toxicity effects impacting CHO cell growth and productivity, in the case of the CHO-DP12s, was not apparent (**Fig. 5B, D** and **E**). Surprisingly, Pb (lead), despite being classed as Class 1 and highly toxic did not exhibit any negative effects on CHO cell growth and viability when screened at 1 mg/L and appeared to be higher in abundance in the non-toxic F-2 film compared to the F-1 film (**Fig. 5A** and **B**). As concentration levels and the thresholds of toxicity would play a role in affecting cell growth, further investigation is needed to determine whether metals such as silver contributed to the toxicity of the F-1 conditioned media as well as determining the exact metal species present within the conditioned media as opposed to the metal salt used during toxicity screening.

bDtBPP endocrine disruption potential testing in vitro

Lastly, leachable compounds from plastic materials have long been on the radar in relation to their safety profiles to human health such as bisphenol A [41]. Not only in the context of their potential toxicity to the bioprocess itself, leachables from the final container closure system, packaging components such as pre-filled syringes and/or processing equipment also pose a threat to the patient [42] if present in the active biopharmaceutical ingredient. Using H295R cells, a range of concentrations of bDtBPP previously observed to negatively impact CHO cell growth [15], 0.005-0.25 µg/mL, was tested in relation to its capacity to disturb hormone production or interact with a range of hormonal receptors. The production of a range of hormones, testosterone, progesterone, estradiol and cortisol was not observed to be induced or retarded by incubation with various concentrations of bDtBPP (**Sup. Fig. 1**). No agonsim was observed in the estrogen, androgen, glucocorticoid and progesterone receptors when tested by reporter gene assays (RGA). However, when bDtBPP was screened by co-incubation with various hormones, testosterone, estrogen, progesterone and cortisol, it was observed that between 0.1-0.25 µg/mL of bDtBPP acts as an androgen antagonist (**Fig. 6B**), thereby inhibiting the natural association of testosterone with its endogenous receptor.

Discussion

Shortly before the discovery of bDtBPP, a means of testing newly developed films for cell culture compatibility was published by Genentech, which monitored the growth of cells in

media that had been stored in 13 different single-use bags [43]. This study highlighted 4 viable bag film options, however, concluded that a "volatile or air-quenched compound, likely generated by gamma irradiation of the problematic bag film" to be the source of their poor performance in cell-based assays. Recently, Dorival-García *et al.,* reported a novel solvent system for the optimised study of highly hydrophobic compounds of which bDtBPP falls under for the identification of potentially non-satisfactory films for cultivation of CHO cell lines [44]. Critically, these studies were performed under realistic possible conditions and determine the milieu of leachables from SUBs that enter the growth media and potentially cause a threat to cell growth. In this instance, in the absence of a benchmark for a "good" performing bag, cell culture media were conditioned by incubation at 37°C in two SUBs made with different plastic films as a means to capture the full leachable profile derived from each film under normal processing conditions and compared to the corresponding control using glass bottles. Two conditioning times were selected in advance; a 7 day time course to best capture the cocktail of leachables derived from these two films in excessive concentrations and; a 3 day time course to capture leachable content over a more modest culture time period.

It was evident that cell culture media conditioned in the older film (F-1) was toxic to CHO cell growth with a negative impact on IgG productivity when incubated for 7 days prior to inoculation. This level of toxicity was not observed in the case of media conditioned in the more modern F-2 film for the same 7-day incubation with cultures performing similar to negative controls and fresh media controls. This suggests that changes have been made in the composition of the plastic materials going from F-1 to F-2 bag generations. Based on reported data, the pre-requisite secondary anti-oxidant, Irgafos® 168, is the origin of the known toxic leachable bDtBPP upon gamma irradiation [45]. Eliminating Irgafos® 168 as a material component during the extrusion process (e.g. high temperature, shear etc.) can dramatically affect polymer degradation [46]. However, minimizing the concentration of Irgafos® 168 and thereby the amount of bDtBPP that can leach has been a proposed alternative for film development and process control [47], which is the case in these two films going from F-1 to F-2. Leachable testing of both F-1 and F-2 film extracts demonstrated that the leachate bDtBPP was present at far higher concentrations in the F-1 condition media compared to the more recent F-2 film (**Fig. 4B and C**), potentially contributing to the cellular toxicity observed in the case of F-1 cultured CHO cells. In relation to the abundance of its pre-requisite Irgafos® 168, this was not detected under any extraction conditions preventing us from concluding that reduced bDtBPP is a result of minimal Irgafos® 168 content. Evidence suggests that it may be degraded

during the incubation step due to oxidation at high temperature and the use of polar extraction solvents [48–50].

Previous studies have demonstrated bDtBPP to leach at concentrations ranging from 0.025-0.11 mg/L in a panel of SUB films over a 4 day conditioning period [14]. Our data indicates that a residency time period of 3 days may not be sufficient enough to leach toxic compounds such as bDtBPP to levels comparable to 7-days. Proteomic profiling of CHO-K1 and CHO-DP12 cells exposed to the toxic F-1 7 day conditioned media revealed several biological processes critical to bioprocess efficiency (**Table 1**). Reduced growth of both CHO cell lines in F-1 conditioned media exhibited a weakened capacity for ATP synthesis through diminished electron transport through the mitochondrial electron transport system as well as an increased response to oxidative stress. Gene ontology analysis of the DE proteins in both CHO-K1 and CHO-DP12 cells cultured in F-1 conditioned media supported this observation with enrichment for biological processes associated with oxidative stress and oxidative phosphorylation (**Table 2**). This weakened mitochondrial activity was highlighted through the reduction in expression of a subset of proteins all involved in NADH dehydrogenase activity (*NDUFS2, NDUFS8, NDUFV1* and *NDUFA10*) [51]. This complex, composed of 47 sub-units (7 mtDNA encoded/40 nDNA encoded), represents the main electron entry point of the ETS as well as contributing substantially to the formation of the proton gradient across the inner mitochondrial membrane, which drives ATP synthesis culminating in the consumption of oxygen and the generation of water [51]. Interrogating this phenotype further determined that the oxygen consumption rate (OCR) of cells cultured in F-1 conditioned media displayed a reduced OCR when compared to negative controls and cells cultured in the non-toxic F-2 conditioned media. Previous studies have demonstrated that CHO cells under a med-high fed-batch culture process exhibited a reduce OCR in combination with a fast-growing phenotype when compare to controls [52]. Furthermore, the extracellular acidification rate (ECAR) was also found to be reduced in F-1 cultured cells exhibiting impeded growth capacity when compared to F-2 cultured cells and negative controls (**Fig. 3C**). Acidification of the culture media is a result of lactate production through glycolytic metabolism and is a metabolic pathway associated with biomass accumulation and exponential cell growth [52].

Cells cultured in F-2 conditioned media maintained a similar glycolytic flux to negative control cells suggesting that the underlying cellular architecture (19 DE proteins common to both CHO-K1 and CHO-DP12s) that is influenced by the potential leachates derived from the F-2 film does not impact critical biological processes. Oxidative stress was another signature

biological process evident in F-1 conditioned media-exposed cells with proteins such as *LONP1* and *VNN1* being increased in abundance. Previous spiking studies using bDtBPP alone revealed oxidative stress through the expression of heme oxygenase 1 (*HMOX1*) further supporting the presence of this leachate in F-1 film conditioned media [15], as shown by LC-MS. The mechanism of action of bDtBPP negatively impacting CHO cell cultures is unknown other than what is indicated through proteomic profiling. However, the chemical composition of bDtBPP itself gives this molecule potentially a high affinity for oxygen therefore scavenging dissolved oxygen within the media resulting in hypoxia, a biological process further highlighted in previous work through the expression of hypoxia upregulated protein 1 (*HYOU1*). A simple spiking study of bDtBPP versus a DMSO control in media resulted in an accelerated depletion of dissolved oxygen when monitored using the Oxygraph-2k respirometer (Data not shown), indicating the potential for this leachable and by association the F-1 conditioned media to induce hypoxia in cultured cells.

Leachables analysis further highlighted a panel of compounds present at higher concentrations in the F-1 film compared to F-2 film extracts again suggesting reduced primary raw materials used in bag manufacturing such as in the case of Irgafos® 168 as indicated by the reduced concentration of bDtBPP (**Fig. 3B and C**). From a panel of trace metals screened, silver (Ag) was the only one found to be toxic to both CHO-K1 and CHO-DP12 cells at high concentrations of 1 mg/L. Despite its toxicity at such high concentrations, silver elicited no growth inhibitory effects at doses as high as 0.25 mg/L (**Fig. 5A and C**). Going from generation F-1 to generation F-2, the levels of Ag within film extracts are reduced but still present (**Fig. 4D**). The poor performance of CHO cells when cultured in F-1 conditioned media could be as a result of the combined effects of toxic leachates such as bDtBPP and Ag, however, further investigation would be required to examine the exact concentrations of leachables that enter the media after 7 days and if these concentrations are in line with cell toxicity assays. It is also important to note that the metal salts used in this screen may not reflect the exact metal species that leaches from these plastic films but remains indicative of potential toxicity with further investigation being require.

Validating the safety profile of the F-2 film over its predecessor F-1 counterpart for CHO cell culture is an important step forward in progressing the adoption of this technology for recombinant protein drug manufacturing within the biopharmaceutical industry. However, from the perspective of human health, most plastic products release estrogenic chemicals [41] highlighting the potential for endocrine disrupting leachates to be present within the media and

possibly persisting through purification processes. We have demonstrated that the well-known leachate, bDtBPP, is considerably reduced in its abundance in the more recent film generation (F-2) but is still present. Using this as an example, it was evident upon screening at a range of concentrations (0.005-0.25 mg/L), as previously reported [15], that bDtBPP possessed the capacity to act as an androgen receptor antagonist (**Fig. 6B**). It has previously been reported that bDtBPP accumulates to levels ranging from 0.025-0.11 mg/L over 3-4 days incubation under bioprocess-relevant conditions [14]. Our data demonstrates that this leachate present in modern generations of films can potentially act as an endocrine disruptor at concentrations reported to leach and accumulate in media extracts. Leachable analysis in this present study was qualitative, allowing for normalised abundances between F-1 and F-2 films and not exact concentrations. Regardless, the potential for additional leachables from SUBs or single-use technologies requires further investigation.

Conclusion

Single-use technologies, in particular SUBs, are gaining wide-spread acceptance within the biomanufacturing space due to more recent generations of films being developed and a better understanding on the safety concerns around leachable materials. We have demonstrated that media conditioned in an older F-1 SUB film inhibited cell growth when compared to controls. Contrary to this, cells cultured in media conditioned using a newer film generation, F-2, reached similar cell densities to control cultures. Poor cell growth was associated with reduced mitochondrial activity as well as glycolytic metabolism in addition to oxidative stress, highlighted through proteomic analysis. Parallel leachable analysis on film extracts used for cell culture assays demonstrated that the abundance of most leachates was reduced when going from one generation to the next including the toxic compound bDtBPP as well as the potentially toxic metal silver (Ag). Other compounds were identified that were unique to the more recent film generation (F-2) which suggests that new material components are being used in film manufacturing and therefore must be subjected to E&L analysis as well as toxicology screening. As the pre-requisite component of bDtBPP, Irgafos® 168, was not detected in either F-1 or F-2 extracts, we were unable to definitively conclude that the reduced levels of bDtBPP leaching was due to reduced Irgafos® 168 starting material. Furthermore, in the case of the toxic F-1 film, there appears to be a time-dependency which allows leachables to accumulate to toxic levels in the case of 7 versus 3-day conditioning studies. This would suggest that older films

like F-1 could be suitable for use in short batch runs such as 48-72 hour seed trains used in scale-up without compromising cell growth whereas longer fed-batch production processes may be more suited to newer films. Finally, after evaluating the safety profile of a modern single-use film for CHO cell culture, we demonstrated that leachables such as the well-known bDtBPP possess the capacity to act as an endocrine disruptor, in particular, an androgen antagonist. This would suggest that further studies should be performed to know, in more detail, the effects such leachates have on the endocrine system and the potential threat to patient safety. Studies such as this support the safe implementation of SUBs within the biopharmaceutical industry and demonstrate that bag manufacturers are tailoring plastic formulations to address past concerns over toxic leachables. Nevertheless, the lack of standardisation and regulation emphasises the necessity to further develop a toolkit for assessing the safety and biocompatibility of emerging films for use in biomanufacturing.

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Conflicts of Interest

The authors declare no commercial or financial conflict of interest.

Abbreviations

bDtBPP - bis(2,4-*di*-*tert*-butylphenyl)-phosphate, CHO – Chinese hamster ovary, DE – Differentially Expressed, E&Ls – Extractables and Leachables, mAb – Monoclonal antibody, PEG – Polyethylene Glycol, SS – Stainless Steel, SUB – single-use bioreactor

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Figure Legends

Figure 1: Impact of conditioned media from two single-use disposable bioreactor bags on the bioprocess-relevant phenotypes of CHO-K1 and CHO-DP12 cells. Cells were cultured in an 8-10 day batch process in chemically defined media (BalanCD CHO Growth A) and analysed every 2 days for cell density and culture viability using the Guava ViaCount Assay as well as for IgG production using ELISA. CHO-K1 cells cultured in media conditioned in the older F-1 film (A and B) and newer F-2 film (C and D) for 7 days. CHO-DP12 Cells cultured in same F-1 (E-G) and F-2 (H-I) media conditioned. Control cells were cultured in media conditioned for the same 7 day timeframe but done so in glass bottles. An additional control "Fresh BalanCD" was included which compares culture media not subjected to the conditioning process. (n = 9, *** $P \leq 0.001$, * $P \leq 0.05$).

Figure 2: Impact of conditioned media from two single-use disposable bioreactor bags on the bioprocess-relevant phenotypes of CHO-K1 and CHO-DP12 cells. Cells were cultured in an 8-10 day batch process in chemically defined media (BalanCD CHO Growth A) and analysed every 2 days for cell density and culture viability using the Guava ViaCount Assay as well as for IgG production using ELISA. CHO-K1 cells cultured in media conditioned in F-1 (A-B) and F-2 (C-D) films for 3 days. CHO-DP12 cells cultured in media conditioned in F-1 (E-G) and F-2 (H-I) films for 3 days Control cells were cultured in media conditioned for the same 3 day timeframe but done so in glass bottles. An additional control "Fresh BalanCD" was included which compares culture media not subjected to the conditioning process. (n = 9, *** $P \leq 0.001$, * $P \leq 0.05$).

Figure 3: The number of both common and unique differentially expressed proteins identified through quantitative label-free LC-MS/MS proteomic analysis from CHO-K1 and CHO-DP12 cells cultured in 7 day F-1 and F-2 film conditioned media and harvested 96 hours into culture for proteomic analysis are represented in the Venn diagram (A). Using the Seahorse FX96 analyser, CHO-K1 cells cultured in 7 day conditioned media in both F-1 and F-2 films were assessed for mitochondrial/TCA cycle activity by monitoring the oxygen consumption rate (OCR), B) as well as glycolytic metabolism through the assessment of the extracellular acidification rate (ECAR), C). Cells were harvested for analysis 96 hours into culture and compared to control negative media cultures. (n = 3).

Figure 4: A) Comparative chart for identified non-volatile leachables in both evaluated SUB models. Comparative LC chromatograms from (B) F-1 and (C) F-2 films. Some

compounds that demonstrated higher levels in F-1 are highlighted, including bDtBPP, and details for these are shown in table 3. D) Comparative chart for identified elemental leachables from both evaluate SUB models. E) Comparative chart for the identified concentrations of elemental leachable in both evaluated SUB models.

Figure 5: Impact of various elemental leachables on the bioprocess-relevant phenotypes of CHO-K1 and the antibody-producing CHO-DP12 cell lines. Cells were cultured in chemically defined (BalanCD CHO Growth A) media spiked on day 0 with the various elemental metal (Al, Pb, Ni, Ag and Na) and harvested 96 hours into culture for growth and viability analysis using the Guava ViaCount Assay and IgG production using ELISA. Metals were dissolved in water and spiked at a concentration of 1 mg/L in both CHO-K1 (A) and CHO-DP12 (C) cells on day 0 of culture and assessed for growth and viability. Various concentrations of silver (Ag) ranging from 0.001-2 mg/L was spiked at day 0 in CHO-K1 (B) and CHO-DP12 (D) cultures and assessed for growth and viability. Additionally, in the case of CHO-DP12 cells, IgG productivity was assessed (E). (n = 3, *** $P \leq 0.001$, * $P \leq 0.05$).

Figure 6: Antagonist effects of bDtBPP (0.005 - 0.25 µg/ml) in the androgen and progestagen responsive TARM-Luc cell line. The responses measured are compared to the solvent control (0.2% DMSO) and the positive control (testosterone 288 ng/ml). Percentage responses are expressed as ±SEM for three independent exposures in triplicate (n=3), P = <0.001 (***).

Supplementary Figure Legends

Supplementary Figure 1: Using ELISA based methods, the average production of the respective steroid hormone A) Testosterone, B) Progesterone, C) Estradiol and D) Cortisol was determined upon incubation bDtBPP over a range of concentrations. (n = 3).

Figure 1

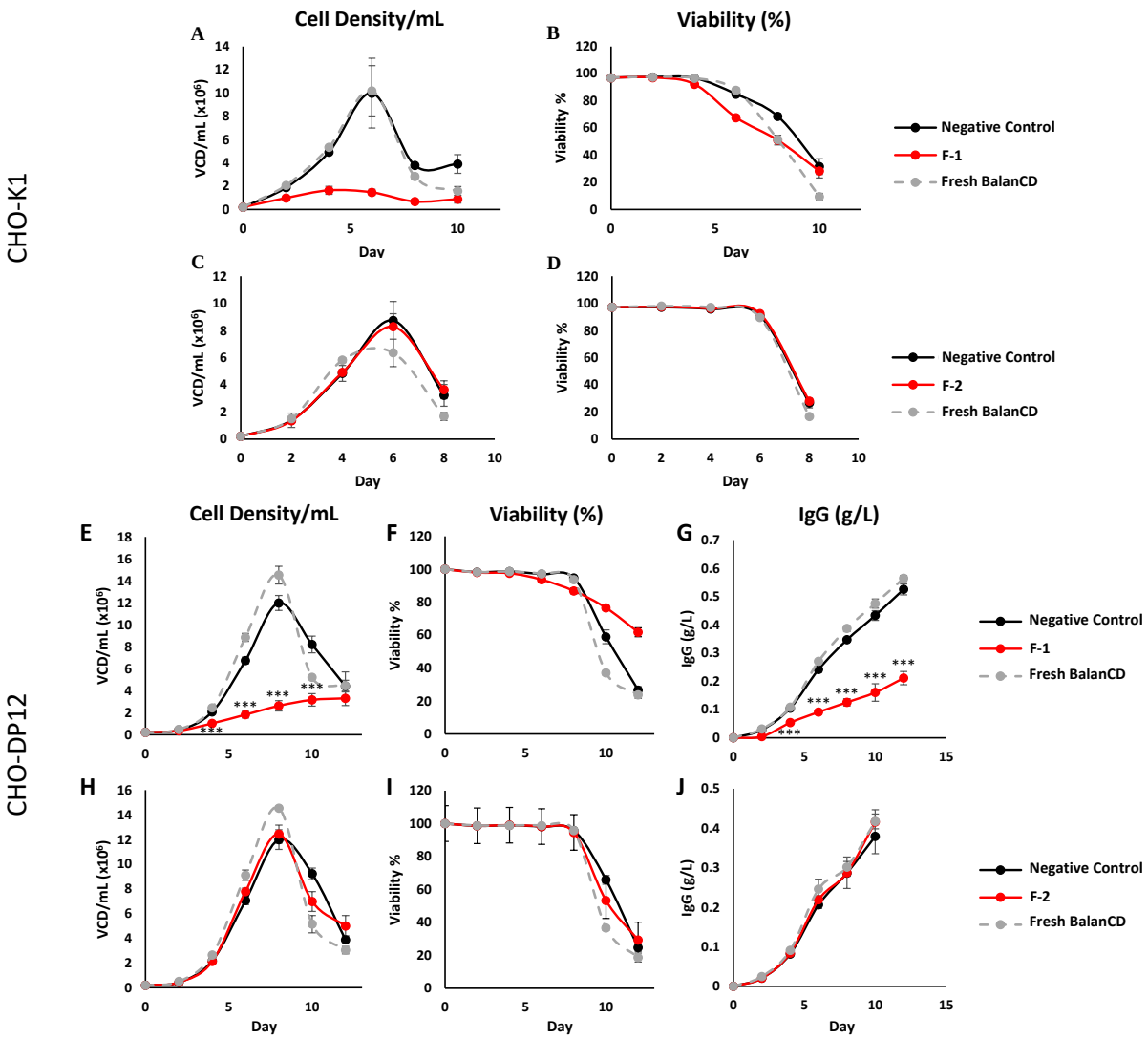
CHO-K1
CHO-DP12
Cell Density/mL
Viability (%)
IgG (g/L)
VCD/mL (x10⁶)
Viability %
IgG (g/L)
Day
A
B
C
D
E
F
G
H
I
J
Negative Control
F-1
F-2
Fresh BalanCD
***

Figure 2

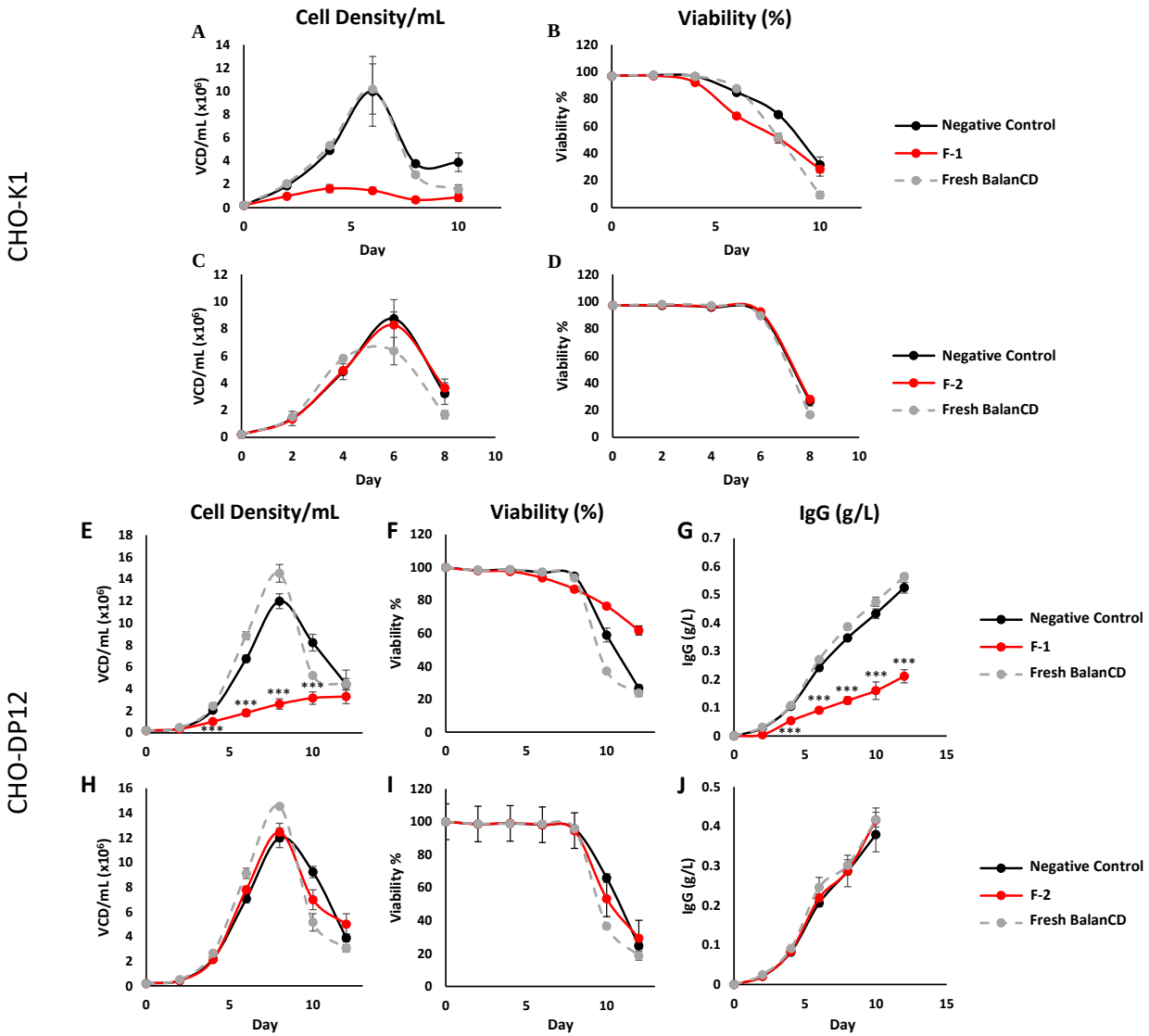
CHO-K1
CHO-DP12
A
Cell Density/mL
VCD/mL (x10⁶)
Day
B
Viability (%)
Viability %
Day
Negative Control
F-1
Fresh BalanCD
C
VCD/mL (x10⁶)
Day
D
Viability %
Day
Negative Control
F-2
Fresh BalanCD
E
Cell Density/mL
VCD/mL (x10⁶)
***
Day
F
Viability (%)
Viability %
Day
G
IgG (g/L)
IgG (g/L)
***
Day
Negative Control
F-1
Fresh BalanCD
H
VCD/mL (x10⁶)
Day
I
Viability %
Day
J
IgG (g/L)
Day
Negative Control
F-2
Fresh BalanCD

Figure 3

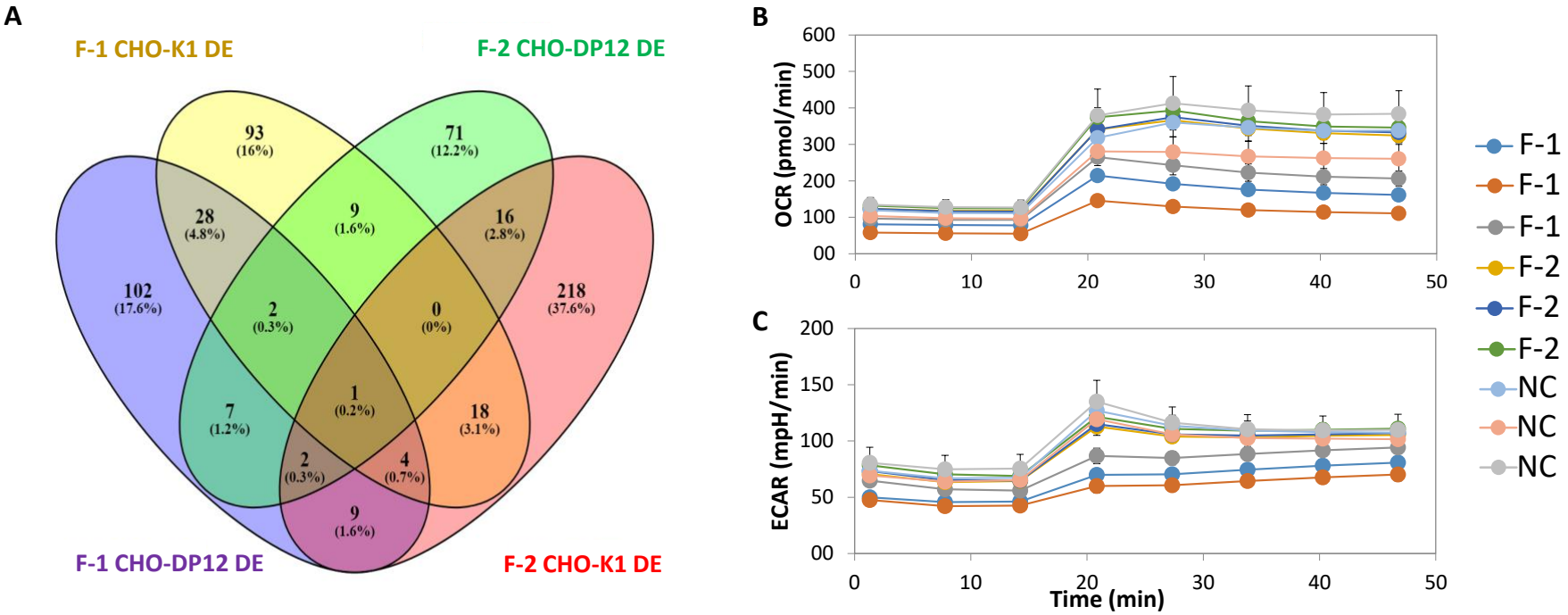
A
F-1 CHO-K1 DE
F-2 CHO-DP12 DE
93
(16%)
71
(12.2%)
28
(4.8%)
9
(1.6%)
16
(2.8%)
102
(17.6%)
2
(0.3%)
0
(0%)
218
(37.6%)
1
(0.2%)
7
(1.2%)
18
(3.1%)
2
(0.3%)
4
(0.7%)
9
(1.6%)
F-1 CHO-DP12 DE
F-2 CHO-K1 DE
B
OCR (pmol/min)
600
500
400
300
200
100
00
0
10
20
30
40
50
C
ECAR (mpH/min)
200
150
100
50
00
Time (min)
F-1
F-1
F-1
F-2
F-2
F-2
NC
NC
NC

Figure 4

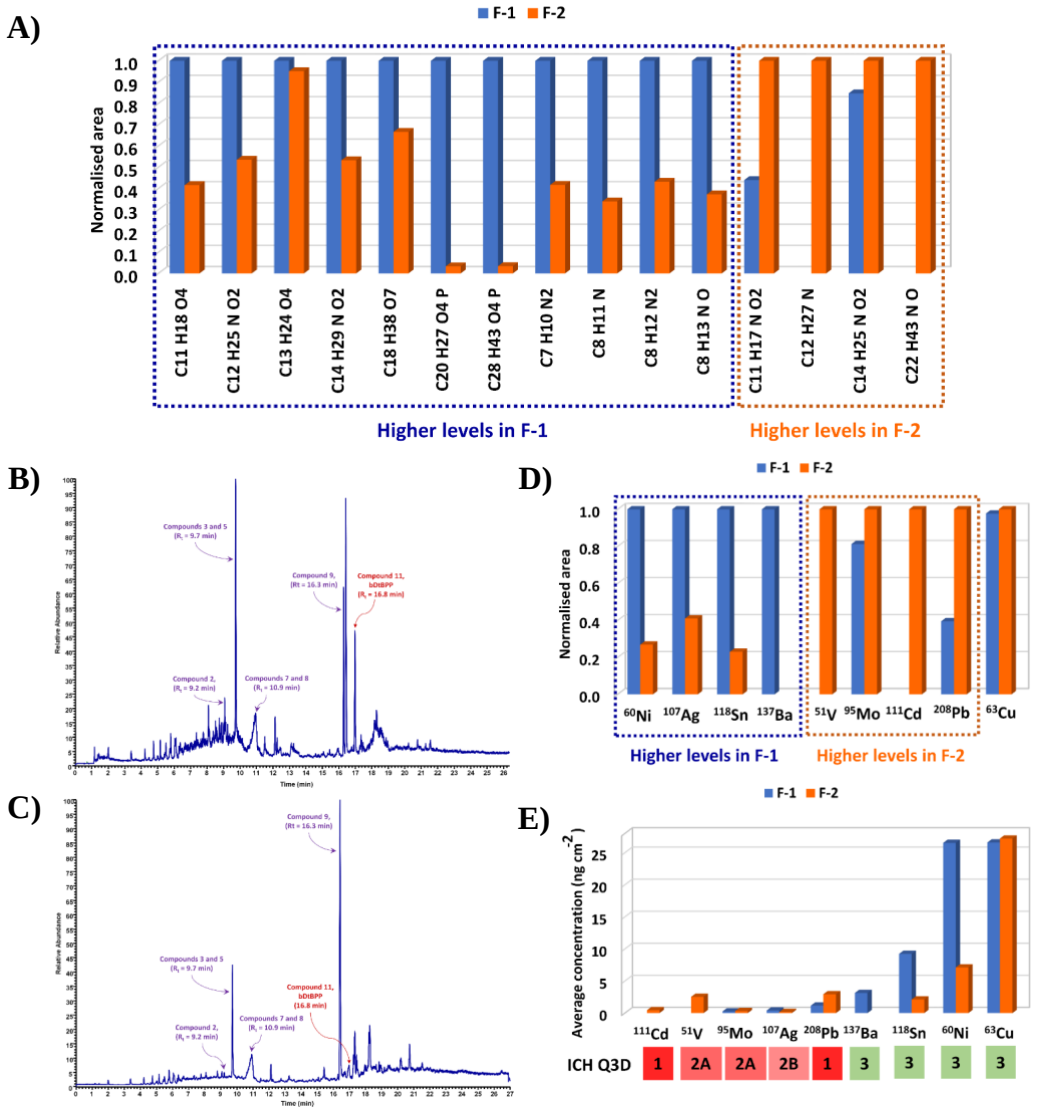
A)
F-1
F-2
Normalised area
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
C11 H18 O4
C12 H25 N O2
C13 H24 O4
C14 H29 N O2
C18 H38 O7
C20 H27 O4 P
C28 H43 O4 P
C7 H10 N2
C8 H11 N
C8 H12 N2
C8 H13 N O
C11 H17 N O2
C12 H27 N
C14 H25 N O2
C22 H43 N O
Higher levels in F-1
Higher levels in F-2
B)
Compounds 3 and 5
Compound 9,
Compound 11,
bDiBPP
Compound 2,
Compounds 7 and 8
Time (min)
C)
Compound 9,
Compounds 3 and 5
Compound 11,
bDiBPP
(16.8 min)
Compound 2,
Compounds 7 and 8
Time (min)
D)
F-1
F-2
Normalised area
1.0
0.8
0.6
0.4
0.2
0.0
60Ni
107Ag
118Sn
137Ba
51V
95Mo
111Cd
208Pb
63Cu
Higher levels in F-1
Higher levels in F-2
E)
F-1
F-2
Average concentration (ng cm-2)
25
20
15
10
5
0
111Cd
51V
95Mo
107Ag
208Pb
137Ba
118Sn
60Ni
63Cu
ICH Q3D
1
2A
2A
2B
1
3
3
3
3

Figure 5

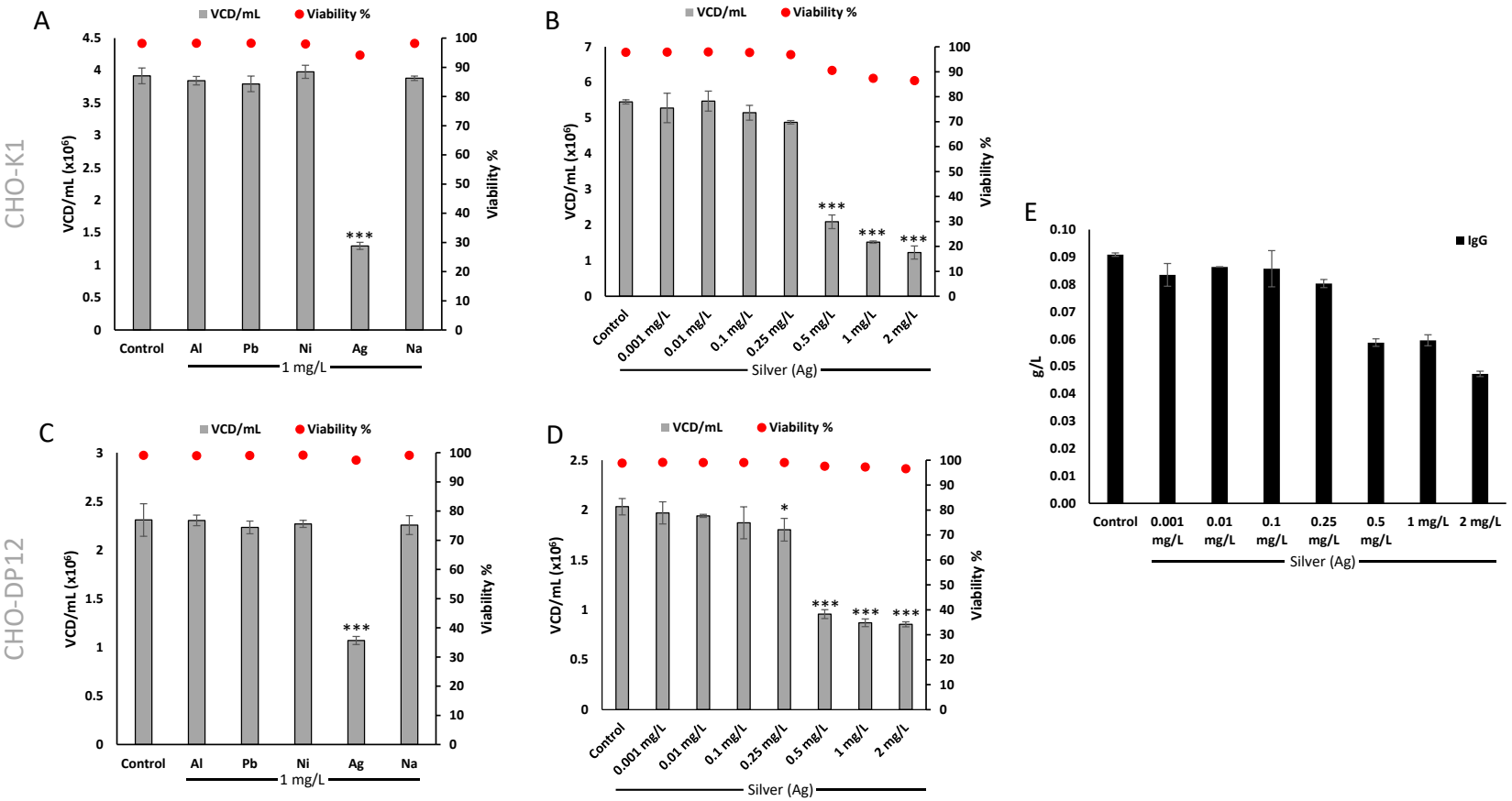
A
B
C
D
E
CHO-K1
CHO-DP12
VCD/mL
Viability %
VCD/mL (x10⁶)
Viability %
Control
Al
Pb
Ni
Ag
Na
1 mg/L
0.001 mg/L
0.01 mg/L
0.1 mg/L
0.25 mg/L
0.5 mg/L
1 mg/L
2 mg/L
Silver (Ag)
IgG
g/L
***
*

Figure 6

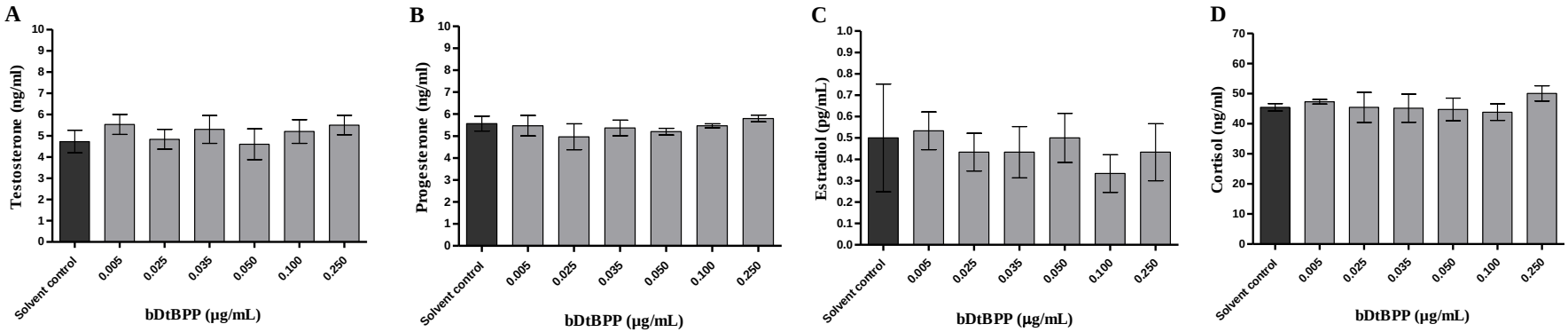
A
Testosterone (ng/ml)
Solvent control
0.005
0.025
0.035
0.050
0.100
0.250
bDtBPP (µg/mL)
B
Progesterone (ng/ml)
bDtBPP (µg/mL)
C
Estradiol (pg/mL)
bDtBPP (µg/mL)
D
Cortisol (ng/ml)
bDtBPP (µg/mL)

Table 1: Common Differentially Expressed proteins in CHO cells incubated in F-1 film conditioned media

Gene I.D.	UR/DR	$FC^{CHO-DP12}$	FC^{CHO-K1}	Biological Process
NDUFS2	DR	4.17	1.51	ATP synthesis
NDUFS9	DR	2.55	1.70	Electron transport
NDUFV1	DR	2.30	1.66	ATP synthesis
NDUFA10	DR	2.17	1.68	Electron transport
PCK1	UR	1.59	2.04	Glucose metabolism
LONP1	UR	1.79	2.14	Oxidative stress
VNN1	UR	1.51	1.62	Oxidative stress
CHCHD1	UR	1.64	1.75	Mitochondrial translation elongation

Abbreviations: CHCHD1 – Coiled-coil-helix-coiled-coil-helix domain-containing protein 2**, DR –** Downregulated**, FC –** Fold Change**, LONP1 –** Lon protease-like**, NDUFS2 –** NADH dehydrogenase iron-sulfur protein 2**, NDUFA10 –** NADH dehydrogenase 1 alpha sub-complex subunit 10**, NDUFS9 –** NADH dehydrogenase iron-sulfur protein 9**, NDUFV1 –** NADH dehydrogenase flavoprotein 1**, PCK1 –** Phosophoenolpyruvate carboxykinase**, UR –** Upregulated**, VNN1 -** Pantetheinase

Table 2: Gene Ontology Analysis for Differentially Expressed proteins from CHO-K1/DP12 F-1 conditioned media

GO Term	GO	P-Value
Oxidation-Reduction process	BP	4.6E-11
Response to oxidative stress	BP	5.2E-5
Mitochondrial respiratory chain complex I	CC	2.0E-13
Mitochondrion	CC	1.1E-9
Oxidative Phosphorylation	KEGG	1.1E-9

Abbreviations: BP – Biological Process**, CC –** Cellular Component**, GO –** Gene Ontology

Table 3
Summary of identified leachables by LC-MS.

	Compound name	Formula	Molecular Weight	Annotation	ΔMass [ppm]	Tentative function	Cramer class	Carcinogenicity alerts In silico[1]	
								A	B
1	N,N-di(2-hydroxyethyl)-p-toluidine	$C_{11}H_{17}NO_2$	195.12611	3077-12-1	0.92	Photopolymerization/adhesive	3	NO	NO
2	1,4-dioxacyclotridecane-5,13-dione	$C_{11}H_{18}O_4$	214.12025	4471-27-6	1.20	Adhesive for multilayer materials	1	NO	NO
	3,3-Dimethyl-1,5-dioxacycloundecane-6,11-dione			94113-47-0		Contamination formed in polyolefin containers	1	NO	NO
3	1,2-amidododecanoic acid	$C_{12}H_{25}NO_2$	215.18841	693-57-2	0.56	Residue from production	1	NO	NO
4	N,N-dimethyldecan-1-amine	$C_{12}H_{27}N$	185.21452	1120-24-7	0.90	Catalyst degradation product	1	NO	NO
5	Ethylmalonic acid dibutyl ester	$C_{13}H_{24}O_4$	244.16699	1113-92-4	1.93	Propylene copolymer, residue from production	1	NO	YES
	Diethyl azelate			624-17-9		Plasticizer	1	NO	NO
6	2-Propenoic acid 2-methyl- 1,2,2,6,6-pentamethyl-4-piperidinyl ester	$C_{14}H_{25}NO_2$	239.18838	68548-08-3	0.62	UV stabilizer	3	NO	NO
7	N-(2-hydroxyethyl)-dodecanamide	$C_{14}H_{29}NO_2$	243.21957	142-78-9	1.07	Antistatic agent	3	NO	NO
8	PPG n6	$C_{18}H_{38}O_7$	366.26130	25322-69-4	1.23	Polymer block degradation product	3	NO	NO
9	2-Ethylhexyldiphenyl phosphate (Santicizer®)	$C_{20}H_{27}O_4P$	362.16400	1241-94-7	1.93	Flame retardand, plasticizer	3	NO	YES
10	cis-1,3-docosenic acid amide (Kemamide® E ultra)	$C_{22}H_{43}NO$	337.33386	112-84-5	1.78	Slip agent	3	NO	NO
11	bis(2,4-di-tert-butylphenyl)phosphate (bDtBPP)	$C_{28}H_{43}O_4P$	474.28925	69284-93-1	1.37	Antioxidant degradation product	3	NO	NO
12	2,6-Toluenediamine	$C_7H_{10}N_2$	122.08440	823-40-5	0.05	Polyurethane intermediate	3	YES	NO
	2,4-Toluenediamine			95-80-7		Polyurethane intermediate	3	YES	NO
13	5-Ethyl-2-methyl-pyridine	$C_8H_{11}N$	121.08917	104-90-5	0.18	Vinyl acetate catalyst/olefin epoxidation catalyst / solvent for polycarbonate polymerisation	3	NO	NO
	2,4-Dimethyl-aniline			95-68-1		Catalyst degradation product	3	YES	NO
14	1,3-Bis(aminomethyl)benzene	$C_8H_{12}N_2$	136.10023	1477-55-0	1.34	Polyamide modification agent	3	NO	NO
	Tetramethyl succinonitrile			3333-52-6		Polymerization initiator degradation product	3	NO	NO
15	1-ethenylazepan-2-one	$C_8H_{13}NO$	139.09981	2235-00-9	0.67	Coating	3	NO	NO
16	Tris(2,4-di-tert-butylphenyl)phosphate	$C_{42}H_{63}O_4P$	662.44640	95906-11-9	1.22	Antioxidant degradation product	3	NA[2]	NA[2]

[1] From Toxtree using Benigni/Biossa rulebase. A = considering genotoxic effects, B = considering non-genotoxic effects. [2] NA = Not available.